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Development of Repressible Systems to Control Gene Expression in Vaccinia Virus

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**Development of Repressible Systems to
Control Gene Expression in Vaccinia Virus**

Allison Titong

B.S., University of California, Davis 2006

B.G.S., University of Connecticut, Storrs 2010

A Thesis

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APPROVAL PAGE

Master of Science Thesis

**Development of Repressible Systems to
Control Gene Expression in Vaccinia Virus**

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ABSTRACT

Two vaccinia virus (VACV) expression systems that contain elements from the lactose (*lac*) and the tetracycline (*tet*) operons of *E. coli* were developed to repress the expression of a reporter gene, enhanced green fluorescent protein (EGFP), in the presence of *tet* operon inducers. In the first system, *lac* and *tet* operon elements were arranged in a gene circuit, and in the presence of increasing concentrations of a *lac* operon inducer (isopropyl- β -D-thiogalactoside, IPTG), EGFP expression increased in a dose dependent manner and at high IPTG concentrations, expression reached the same levels as a positive control virus. Importantly, in the presence of increasing concentrations of the *tet* operon inducer (doxycycline, DOX), EGFP expression decreased in a dose dependent manner and at high concentrations of DOX, expression was repressed to the degree observed in a negative control virus that does not express EGFP. In the second system, recombinant VACVs constitutively expressing six mutant versions of the *tet* repressor gene (*tetR*) shown to bind *tet* operators in the presence, but not absence of inducers (reverse *tetR* genes), were developed. In the presence of tetracyclines (TCs), the recombinant VACVs exhibited various degrees of repression of EGFP expression, with increasing concentrations of TCs leading to EGFP repression in a dose dependent manner, and in some instances, down to the degree observed in a negative control virus. In light of the renewed interest for the use of VACV as vaccine and therapeutic cancer vectors, the repressible VACV expression system developed here can be used to tightly regulate genes essential for VACV replication, thus functioning as a built-in safety mechanism to conditionally control viral replication.

CHAPTER 1

INTRODUCTION

1.1. VACCINATION AND VACCINIA VIRUS

In 1796, Dr. Edward Jenner demonstrated that protection could be achieved against the highly pathogenic variola virus (smallpox virus) by prior infection with a related and less pathogenic virus, cowpox virus. Today, this preventative measure is called vaccination. Between the 19th and 20th centuries, it is theorized that vaccinia virus (VACV) replaced cowpox as a result of rudimentary smallpox vaccination practices (Fenner and World Health Organization, 1988). In the 1960s, to finally quell the rampant spread of smallpox disease that had up to a 30% case fatality rate (Fenner and World Health Organization, 1988), the World Health Organization (WHO) launched the smallpox eradication campaign, in which VACV was widely produced and administered as a live vaccine. In 1979, the WHO succeeded in this feat and smallpox virus was declared eradicated (Wehrle, 1980, World Health Organization. Global Commission for the Certification of Smallpox Eradication, 1980). To date, there has been no re-emergence of smallpox and its eradication is considered one of the greatest achievements in the history of medicine.

VACV belongs to the family *Poxviridae* and has many unique characteristics. This large complex virus is roughly 200×250 nm in size and consists of a dsDNA genome that is about 190 kb with nearly 200 open reading frames (ORFs) (Moss and Earl, 2001). Viral replication in VACV is distinctive as its genome encodes its own transcription machinery that allows transcription to occur autonomously within the host cell cytoplasm, with no evidence of viral gene integration into the host genome (Figure 1.1). Additionally, transcription occurs in an ordered cascade of early, intermediate and late transcriptional events controlled by specialized VACV promoters. VACV promoters

have two conserved sequence components, the core and the initiator, that differ between each temporal promoter. Early mRNAs are detected 20 min post-infection (PI) and levels peak within the first 100 min. Intermediate mRNAs are detected within 100 min PI and peaks at about 2 hr PI. Late mRNAs are detected at 140 min PI and increase continuously for about 48 hr. Posttranscriptional modifications of viral mRNA such as capping and polyadenylation occurs, but there is no RNA splicing as viral mRNA is made in the cytoplasm. Subsequently, viral mRNAs are translated by host ribosomes. The assembly of viral genome and proteins occur in viral factories following complex morphogenesis and maturation steps that ultimately lead to the generation of new virions. Mature virions can stay cell associated, bud, or lyse out of the infected host cells (Moss and Earl, 2001).

1.2. VACCINIA VIRUS AS A VECTOR

With the eradication of smallpox, VACV became one of the best characterized viruses and has gained much attention in the fields of vaccines and cancer therapies. In addition to having a wide mammalian host range, VACV is easy to genetically manipulate (Mackett et al., 1982), is able to carry at least 25 kb of heterologous DNA while still maintaining its infectivity (Smith and Moss, 1983), and it is able to elicit potent humoral and cell-mediated immune responses against heterologous antigens (Tartaglia and Paoletti, 1988). Successful VACV-vectored vaccines have been generated (Verardi et al., 2012). For example, a recombinant VACV vaccine expressing the glycoprotein of rabies virus has been used extensively in Europe and North America to eradicate sylvatic rabies (Pastoret and Brochier, 1996). Also, a recombinant VACV-

vectored vaccine expressing the fusion and hemagglutinin glycoproteins of rinderpest virus elicited long-term protective immunity against rinderpest in cattle (Verardi et al., 2002, Yilma et al., 2003). VACV vectored human vaccines for human immunodeficiency virus (HIV) (Harari et al., 2008, McCormack et al., 2008, Bart et al., 2008, Garcia et al., 2011, Goepfert et al., 2011), malaria (Sheehy et al., 2011, Sheehy et al., 2012), and anthrax (Merkel et al., 2010) are currently undergoing pre-clinical evaluation or have reached the stage of human clinical trial testing.

Another unique characteristic of VACV is its tropism for cancer cells. VACV has a predilection to replicate in cancerous cells that can be exploited as delivery systems (e.g., carrying co-stimulatory molecules to induce immune responses to cancer cells) or as oncolytic vectors that can lead to the destruction of cancer cells. Currently, cancer immunotherapies against prostate cancer or oncolytic therapies against solid tumors are being closely evaluated in clinical trials for efficacy (Verardi et al., 2012). Furthermore, a dual-functioning oncolytic recombinant VACV expressing the co-stimulatory molecule granulocyte-macrophage colony-stimulating factor (GM-CSF) is being studied in human clinical trials as an “armed” oncolytic therapy against cutaneous melanoma (Mastrangelo et al., 1999, Kirn and Thorne, 2009, Breitbach et al., 2011, Guse et al., 2011).

1.3. SMALLPOX VACCINE

Despite the eradication of smallpox, there are currently significant concerns that may require widespread smallpox vaccination to resume. For example, one major concern is the threat of smallpox as a bio-weaponized pathogen (Breman and Henderson, 1998). Unaccounted sources of variola virus may still exist around the globe and

contribute to this threat. Additionally, advancements in genetic engineering may allow the laboratory generation of the virus based on published variola virus genomic sequences (Massung et al., 1994). Also, there could be a release of similar or genetically engineered poxviruses that are more pathogenic and virulent (Gottschalk and Preiser, 2005). Another concern is the natural re-emergence of a smallpox-like disease caused by a related virus, such as monkeypox virus (Reynolds et al., 2012). Monkeypox virus is currently endemic in Africa, but a US outbreak in 2003 due to the importation of African rodents highlights its potential to re-emerge as a serious public health hazard outside Africa (Centers for Disease Control and Prevention (CDC), 2003, Reed et al., 2004). Thus, to prepare for these potential risks, the US has a stockpile of a smallpox vaccine (Greenberg and Kennedy, 2008, Monath et al., 2004, Nalca and Zumbrun, 2010).

1.4. ADVERSE EVENTS AND CONTRAINDICATIONS

The smallpox vaccine used in the US during the smallpox eradication campaign (Dryvax, one of the first-generation smallpox vaccines) was prepared from scarified lesions on calves and it was very efficacious. However, a large subset of the population is currently deemed contraindicated against live, replication-competent VACV vaccines. Individuals that are immunosuppressed (HIV/AIDS, transplant, and cancer patients), have (or had) atopic dermatitis and other skin conditions, that are pregnant, or have cardiac problems, are especially susceptible to a range of mild to severe adverse events (Casey et al., 2005, Lane and Goldstein, 2003, Thomas et al., 2008). Mild adverse events include headache, fever, and pain at the inoculation site. However, severe adverse events include eczema vaccinatum (in which VACV replicates in areas affected or once affected by

atopic dermatitis, possibly leading to death), progressive vaccinia (a severe adverse event that usually affects those that are immunosuppressed as the virus grows uncontrollably in flesh, skin and even bones, and could also lead to a grave outcome), and encephalitis (where VACV enters the nervous system, which typically leads to death). In addition, vaccinated individuals can inadvertently inoculate themselves (autoinoculation) or inoculate others via contact transmission. A conservative estimate of individuals in the US that fall within this population is about 25% (Kemper et al., 2002). Affected individuals, depending on the severity of complications, can be treated with vaccinia immune globulin (VIG) or antiviral agents such as cidofovir, ST-246, and some tyrosine kinase inhibitors, but the efficacy of these treatments is questionable (Lane and Goldstein, 2003, Centers for Disease Control and Prevention (CDC), 2009).

There have been great strides taken to increase the safety of smallpox vaccines for individuals with contraindications (Verardi et al., 2012). For instance, a second-generation smallpox vaccine (ACAM2000, a single-clone derivative from the Dryvax vaccine) was produced in cell culture. While ACAM2000 has improved sterility and is the current US stockpiled smallpox vaccine, its safety in terms of adverse events was not improved. In another attempt to increase the safety profile of smallpox vaccines, the VACV strain Ankara was passaged over 570 times in chick embryo fibroblasts. The resulting virus, named Modified Vaccinia Ankara (MVA), is highly attenuated in mammalian hosts. MVA-BN is a prospective third-generation smallpox vaccine developed by the vaccine company Bavarian Nordic that is currently on fast-track human clinical trials. However, the protection afforded by MVA as a smallpox vaccine is uncertain since vaccinated individuals were never exposed to smallpox. Genetic

engineering has also been used as a tool for the generation of attenuated VACV strains such as NYVAC, derived from the Copenhagen strain in which 18 nonessential genes were deleted. NYVAC is also highly attenuated with a restricted host range. Although these attenuated strains provide a better safety profile, they have numerous limitations. In particular, highly attenuated strains may require multiple inoculations to elicit a robust immune response, which is not ideal in the event of the emergence of an orthopoxvirus or a bioterrorist event (Lane, 2011). Furthermore, they incur higher costs to propagate and manufacture and as oncolytic therapies, highly-attenuated VACV strains would not be appropriate, as there is no oncolytic potential in replication-incompetent viruses (Kirn and Thorne, 2009).

1.5. SIGNIFICANCE

VACV, as a replication-competent virus, has several excellent characteristics that make it a powerful smallpox vaccine, as well as a vaccine and therapeutic cancer vector. However, adverse events associated with uncontrollable VACV replication must be addressed. Thus, an approach to address this limitation is to develop a repressible system to control gene expression in VACV. The focus of this project is to develop and test repressible expression systems in VACV by using elements from the tetracycline (*tet*) and lactose (*lac*) operon systems of *E. coli* to control the expression of a reporter gene, enhanced green fluorescent protein (EGFP). A future step would be to place a gene that is essential to VACV viral replication under the control of this repressible system, so that should an adverse event occur after vaccination or treatment, the expression of the

essential gene could be repressed, thereby stopping viral replication and most importantly, the adverse event.

1.6. HYPOTHESIS

The following specific aims will test two different strategies to develop a repressible VACV expression system, so that in the presence of an operon inducer, the gene of interest will be repressed (not expressed):

Specific Aim 1: To develop a repressible VACV expression system assembled via a gene circuit with natural repressors from the *lac* and *tet* operons.

We will develop a recombinant VACV (vIRG, *y*accinia virus *lacI/tetR* Repressible *G*reen fluorescent gene) expressing the natural tetracycline repressor gene (*tetR*) under the control of a constitutive early/late VACV promoter ($P_{E/L}$), and the *lacI* gene under the control of an engineered $P_{E/L}$ VACV promoter containing a *tet* operator (*tetO*) sequence (Figure 1.2). *EGFP* will be under the control of an engineered late VACV promoter (P_{11}) containing a *lacO* sequence. In the absence of tetracyclines (TCs), the TetR repressor binds to the *tetO*₂ operator sequence and prevents the transcription of *lacI*, allowing the expression of EGFP. In the presence of TCs, TetR undergoes a conformational change and does not bind to the *tetO*₂ sequence, allowing LacI to be expressed and to bind to the *lacO* sequence, preventing the expression of EGFP.

Specific Aim 2: To develop a repressible VACV expression system assembled with reverse *tet* repressor mutants and other elements from the *tet* operon.

We will develop recombinant VACVs (vRG, vaccinia virus Repressible Green fluorescent gene) expressing reverse mutants of *tetR* (*revtetR*) under the control of the constitutive P_{EL} promoter, and EGFP under the control of an engineered P₁₁ promoter containing a *tetO*₂ sequence. In the absence of TCs, revTetR does not bind to the *tetO*₂ operator sequence and allows transcription of EGFP (Figure 1.3). In the presence of TCs, revTetR undergoes a conformational change and binds to the *tetO*₂ sequence, preventing the expression of EGFP.

1.7. THE LACTOSE OPERON

The lactose (*lac*) operon is composed of a set of genes that enable prokaryotes to use lactose as a carbon source. The regulatory components of the *lac* operon include a lactose repressor gene (*lacI*), DNA operator sequences (*lacO*), and an inducer, such as the natural inducer (allolactose) or the synthetic analog isopropyl-1-thio- β -D-galactoside (IPTG) (Figure 1.4). The *lacI* gene encodes a homotetrameric LacI repressor protein. Each monomer contains a DNA binding domain, a hinge region, and an inducer binding region. There are three different *lacO* sequences (*O*₁, *O*₂, and *O*₃), with *O*₁ having the highest affinity for LacI. In 1961, Jacob and Monod elucidated the mechanism of the *lac* regulatory system in *E. coli* (Jacob and Monod, 1961). In this system, *E. coli* will use glucose as its primary carbon source. In this case, LacI is expressed constitutively at low levels and negatively controls genes within the *lac* operon by binding to operator

sequences within their independent promoters, thereby leading to the repression of genes that encode β -galactosidase (*LacZ*), permease (*LacY*), and acetyl-transferase (*LacA*). However, in the absence of glucose and in the presence of lactose, inherent signals will initiate the induction of the *lac* operon. First, lactose becomes converted to its isomer, allolactose, which then binds LacI, inducing a conformational change so that LacI no longer binds to the operator sequences (Lewis, 2005, Wilson et al., 2007). RNA polymerase is then able to bind to the promoter regions that overlap the operators and allow the transcription of the genes involved in lactose metabolism (Lewis, 2005, Wilson et al., 2007).

1.8. ALLOLACTOSE ANALOGS

In the history of genetics, the *lac* operon system was one of the first and best studied genetic systems. The main way to study the induction of the *lac* operon was to measure the induced expression of the enzyme β -galactosidase. Before the 1950s, the only known methods of measuring β -galactosidase activity were laborious and there was a need for a more efficient assay. Thus, one of the pioneers in the field of genetics, Joshua Lederberg, collaborated with chemists to synthesize a substrate for β -galactosidase. One colorless and inexpensive substrate, O-nitrophenyl- β -D-galactosidase (ONPG), was catalyzed by β -galactosidase into a yellow product that could be quantitated using a colorimeter. However, ONPG was not an inducer of the system (Müller-Hill, 1996). In a quest to find an inducer that activated the *lac* operon without being metabolized by β -galactosidase, Jacob and Monod also collaborated with chemists to synthesize new inducers. Of all analogs synthesized, methyl-1-thio- β -D-galactoside

(TMG) and isopropyl-1-thio- β -D-galactoside (IPTG) were tested and found to be strong inducers without being metabolized. IPTG was the strongest inducer and when high concentrations of IPTG (5 mM) were used, β -galactosidase was induced in prokaryotes within 3 min (Müller-Hill, 1996). Today, IPTG is commonly used in *lac* inducible expression systems *in vitro* and *in vivo* to initiate transcription of genes of interest.

1.9. USING THE LACTOSE OPERON TO CONTROL GENE EXPRESSION

The *lac* system can also be used as a tool to express genes of interest and it has been adapted for use both *in vitro* and *in vivo*. In 1987, Hu and Davidson successfully developed an inducible system in mammalian cells (mouse L cells), using a plasmid containing a reporter gene, chloramphenicol acetyltransferase (CAT), under the control of an engineered early promoter from the Simian Virus 40 (SV40) containing a synthetic *lacO* analog sequence and using IPTG (synthetic allolactose analog) to induce CAT expression (Hu and Davidson, 1987). Similarly, Brown et al. studied a *lac* inducible system in HeLa cells, in which IPTG was sufficient to induce the expression of CAT (Brown et al., 1987). Cronin et al. adapted the *lac* system to transgenic mice, allowing the inducible expression of a reporter gene in mice receiving 2 mg/ml of IPTG in drinking water (Cronin et al., 2001). More relevant to this project, Fuerst et al. adapted the *lac* operon to VACV by generating inducible recombinant VACVs that contained a reporter gene (β -galactosidase) under the control of a late VACV promoter (P_{11}) with a synthetic *lacO* placed immediately downstream (Fuerst et al., 1989). The maximal expression of β -galactosidase in cell culture was induced with 5 mM IPTG (Fuerst et al., 1989).

1.10. THE TETRACYCLINE OPERON

Transposon *Tn10* of bacteria confers resistance to TCs. The regulatory components of the *tet* operon include a tetracycline repressor gene (*tetR*), operator sequences (*tetO*), and an inducer (TCs) (Figure 1.5). The *tetR* gene encodes a homodimeric TetR repressor protein, where each monomer contains a DNA binding domain, a hinge region, and an inducer binding region. There are two different DNA operator sequences, *tetO*₁ and *tetO*₂, with *tetO*₂ having the highest affinity for TetR. In the absence of TCs, TetR autoregulates its expression and negatively controls the genes within the *tet* operon by binding to the *tetO* sequences that overlap the *tet* operon promoters, thereby leading to the repression of genes that encode TetR and the anti-porter transmembrane protein complex (TetA) (Hillen and Berens, 1994). In the presence of TCs, TetR undergoes a conformational change and no longer binds to the operator sequences (Hillen and Berens, 1994). RNA polymerase is then able to bind to the promoter regions, allowing the expression of TetA, which aids in the efflux of TCs.

1.11. TETRACYCLINE DRUG CLASS DERIVATIVES

TCs are produced by microbes and are known to inhibit translation of other prokaryotes to establish a territorial advantage (Greenwald et al., 2001). The primary mechanism of action of TCs is to selectively block the acceptor site (A-site) of prokaryotic ribosomes, thus preventing the linkage of a new amino acid to the nascent polypeptide chain and ultimately leading to demise of the cell. The *tet* operon is an adaptive genetic mechanism that leads to TC removal so that prokaryotes can resist its

negative effects. TC derivatives have been synthesized in which drugs within this class share the same backbone and differ mostly in functional groups (Greenwald et al., 2001). Of the drugs within this class, the synthetic TCs minocycline and doxycycline (DOX) are widely used in medicine, animal health, and agriculture. However, due to multi-drug resistance, newer derivatives such as glycylcyclines are being investigated *in vitro* and *in vivo*. Another tetracycline widely used in prokaryotic expression systems is anhydrotetracycline (ATC), which has increased affinity for TetR and minimal antibacterial activity.

1.12. USING THE TETRACYCLINE OPERON TO CONTROL GENE EXPRESSION

Like the *lac* operon, the *tet* operon has been used to generate inducible systems to control the expression of a gene of interest. In 1988, Gatz and Quail first adapted the *tet* operon to tobacco plant cells by cotransfecting a plasmid expressing the reporter gene CAT under the control of the cauliflower mosaic virus (CaMV) 35S promoter with two *tetO₂* sites, and another plasmid expressing *tetR* under the constitutive control of CaMV 35S.

In a design of a new mammalian inducible system, Gossen et al. randomly mutagenized the *tetR* gene and screened for mutants that yielded the reverse phenotype in the presence of DOX. One reverse *tetR* mutant characterized contained the amino acid mutations E71K, D95N, L101S, and G102D (Gossen et al., 1995). Then, a reverse tetracycline transactivator (rtTA) was generated by fusing the reverse tetracycline repressor gene (*revtetR*) with a transcriptional activator (TA) from the VP16 gene of

herpes simplex virus. In the presence of TCs, rtTA binds to the *tet* responsive element (TRE), a modified early CMV promoter that lacks strong enhancer elements (P_{minCMV}) and contains seven repeats of *tetO*₂, promoting transcription of a gene of interest. This TC-inducible system, referred to as the Tet-On system, became a useful tool for inducible control of gene expression in eukaryotic cells and transgenic mice.

In 2000, Urlinger et al. wanted to improve the original rtTA system as it was not very sensitive to DOX and had a high basal level of transcriptional activity in the absence of DOX (Urlinger et al., 2000). The *rtTA* gene was subjected to direct and random mutagenesis and six novel mutants (with two to five amino acid changes) that exhibited the reverse TetR phenotype were obtained. Mutant rtTA-S3 (T26A and D95G) resulted in somewhat lower background levels of expression, but in the presence of inducer a reporter gene (luciferase) was expressed at lower levels than the original rtTA. Mutant rtTA-S2 (E19G, A56P, D148E, and H179R) had a similar activation potential as rtTA, but a significantly lower background activity. The mutational contributions in rtTA-S2 were explored by generating mutants rtTA-19/56 (E19G and A56P) and rtTA-148/179 (D148E and H179R). rtTA-19/56 had a stringent reverse phenotype and minute background activity, while mutant rtTA-148/179 was found to have lost the reverse phenotype. To increase the sensitivity of the system to low concentrations of DOX, rtTA-19/56R mutants were grown in media containing 10 ng/ml DOX (less than the initial dose used of 10 µg/ml) and subjected to additional mutagenesis. Mutant rtTA-M1 (S12G, E19G, and A56P) was recovered with increased sensitivity to DOX and a stringent reverse phenotype. The mutant rtTA-M2 was generated to have enhanced activation and high sensitivity to DOX, which had a combination of mutations from

rtTA-148/179R and rtTA-M1. rtTA-M2 had stringent reverse phenotype, low background expression properties, and a higher sensitivity towards DOX, and is currently a useful tool for inducible control of gene expression, especially in transgenic mice. However, a *tet* system utilizing the VP16 transactivator with a minimal CMV promoter would not function in VACV, as VACV transcription is driven by inherent VACV promoters.

Other *revTetR* mutants were also explored by Scholz et al. with the goal to find the minimum number of amino acids required to reverse the phenotype of TetR in *E. coli* (Scholz et al., 2004). The *tetR* gene was randomly mutagenized and selected using a TC-inducible β -galactosidase expression system where in the absence of inducer, revTetR cannot bind to its operator and therefore allows expression of the reporter gene, while in the presence of inducer, revTetR represses the expression of β -galactosidase. This system was adapted to be dependent on ATC, a TC that does not confer antibiotic activity. A total of 41,000 mutants were screened, of which 112 mutants exhibited the revTetR phenotype. Ultimately, two different revTetR mutants having a single amino acid mutation (L17G or V99E) were found. Both revTetR mutants resulted in the expression of approximately 90% β -galactosidase activity in the absence of the inducer (ATC) and only about 3% β -galactosidase activity in the presence of the inducer.

In 2000, Traktman et al. developed TC-inducible VACVs by inserting a *tetO* operator sequence between the transcriptional and translational start sites of VACV genes, enabling their expression to be tightly regulated by TCs (Traktman et al., 2000). Traktman and others have used the TC-inducible system as a tool for regulation of specific VACV genes, thereby leading to a better understanding of basic biological

properties of VACV. Weber, Verardi et al. (unpublished data) used a VACV TC-inducible system developed in the Verardi Lab for a more practical application as a built-in safety mechanism for VACV based on the inducible expression of interferon- γ (IFN- γ). The IFN- γ gene acts as a safety gene *in vivo* when expressed by VAVC, attenuating the virus by more than a million-fold. Immunodeficient mice inoculated with high doses of VACV constitutively expressing IFN- γ are able to rapidly and completely clear infection (Legrand et al., 2005). In this new application of the inducible system, TetR (the repressor) is constitutively expressed and binds to an operator (*tetO₂*) placed downstream of a VACV promoter (Weber et al., 2007), blocking transcription and expression of the IFN- γ gene. Thus, in the absence of TCs, VACV replicates normally, eliciting full immune responses and retaining its oncolytic potential. When complications arise or are suspected, TC treatment causes TetR to undergo a conformational change so that it no longer binds to the operator. This unblocks the promoter and allows expression of IFN- γ , resulting in complete clearance of the virus. Since this inducible built-in safety mechanism is based on the expression of a cytokine (IFN- γ), which has the potential to cause untoward effects, an alternative built-in safety mechanism is greatly needed. Consequently, the development of repressible systems in VACV could provide a built-in safety mechanism that addresses these concerns by allowing us to repress the expression of VACV genes essential for viral replication.

1.13. FIGURES

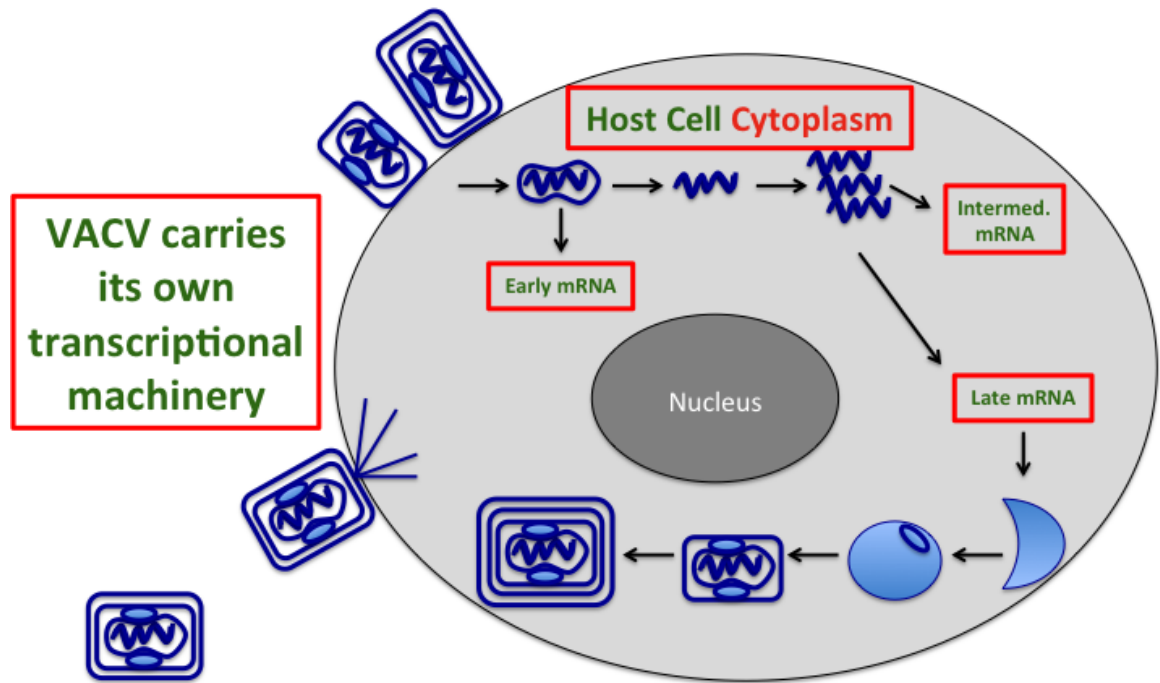


FIGURE 1.1. Simplified VACV replication cycle. Once VACV enters the host cell, an ordered cascade of early, intermediate, and late transcriptional events occur that lead to the morphogenesis and maturation of progeny viral particles (virions).

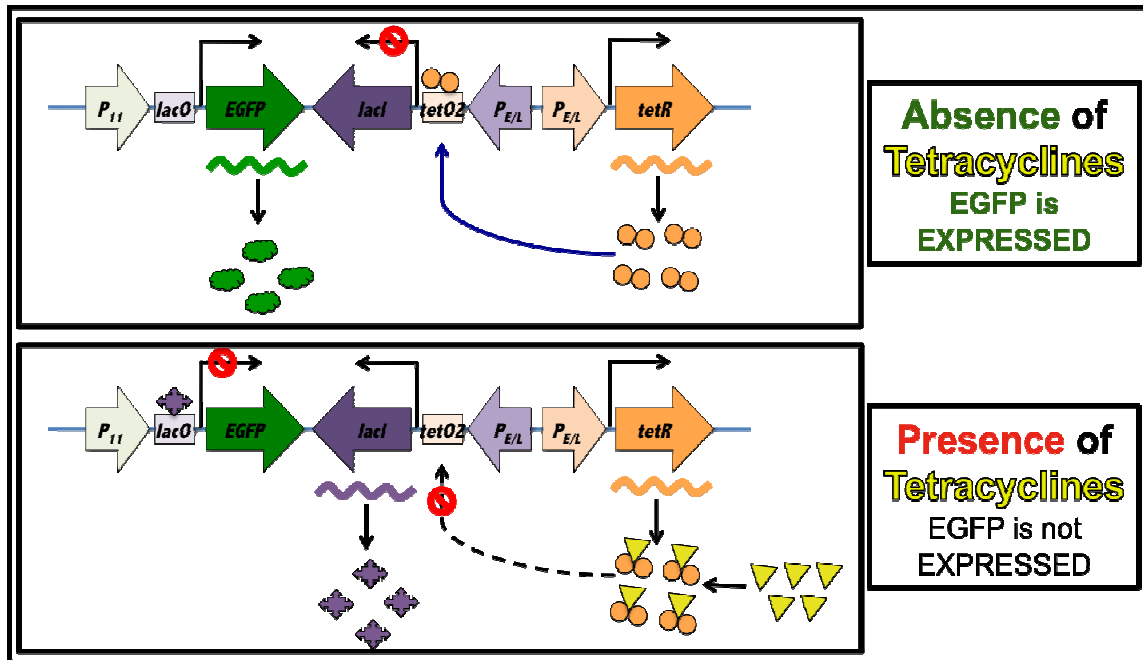


FIGURE 1.2. Tetracycline-controlled repression of EGFP expression in the vIRG system (*lacI/tetR* gene circuit). The *tetR* gene is transcribed constitutively (under $P_{E/L}$) and in the absence of TCs, TetR binds to *tetO2*, blocking expression of *lacI* by the upstream $P_{E/L}$ promoter and allowing EGFP expression. In the presence of TCs, TetR undergoes a conformational change that does not allow it to bind onto the operator and block *lacI* expression. The LacI repressor is then made and it blocks the transcription of EGFP by binding onto the *lacO* sequence downstream from the P_{I1} promoter.

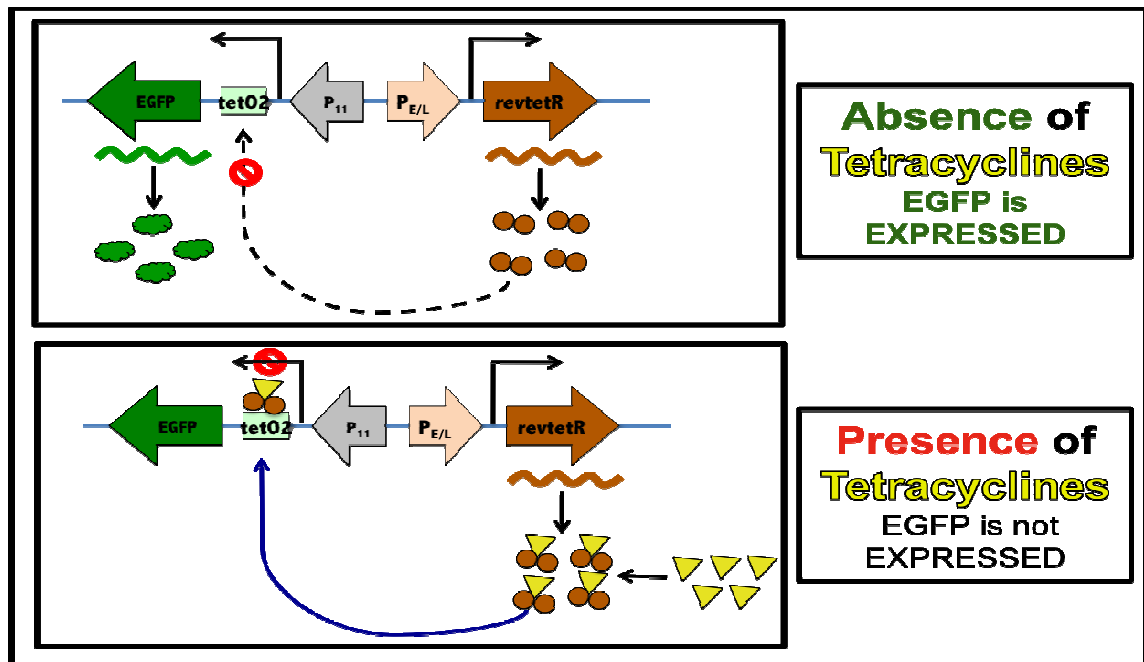


FIGURE 1.3. Tetracycline-controlled repression of EGFP expression in the vRG system (revTetR). In the absence of TCs, revTetR does not bind to *tetO2*, allowing expression of EGFP. In the presence of TCs, revTetR undergoes a conformational change that allows it to bind onto the *tetO2* operator, blocking the transcription of EGFP from the upstream P_{11} promoter.

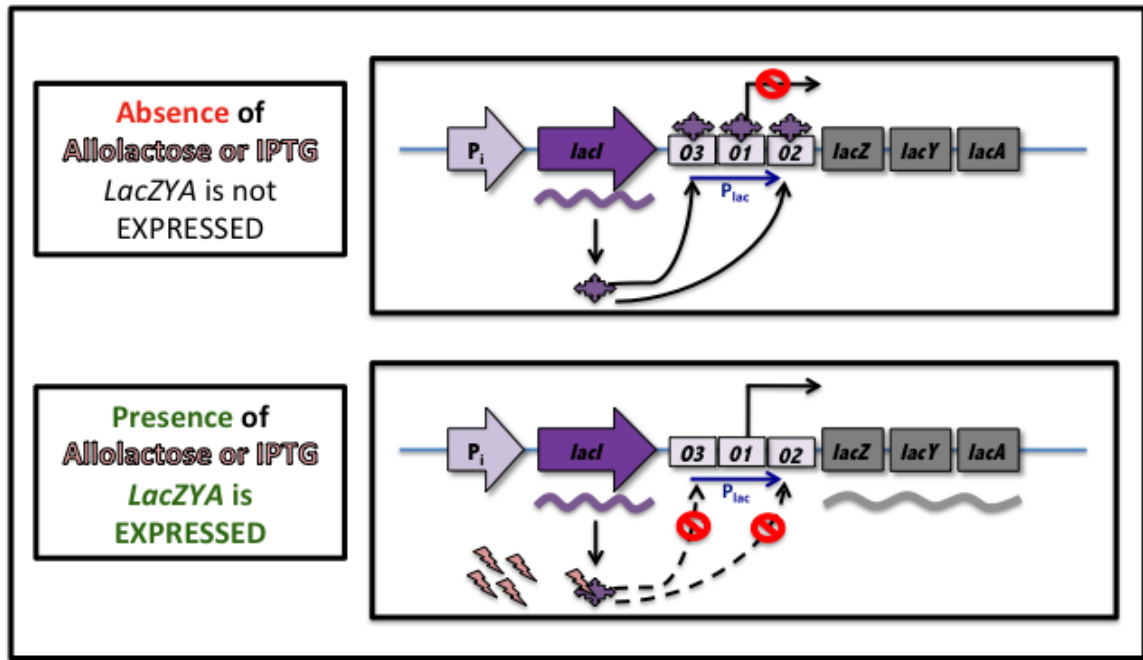


FIGURE 1.4. Genetic mechanism of the *lac* operon. In the absence of inducer, LacI repressor proteins bind onto the operator regions to negatively control the expression of *lacZ*, *lacY*, and *lacA*. In the presence of inducer, LacI undergoes a conformational change so that it can no longer bind to the *lacO* operators, thus allowing the expression of the genes required for the metabolism of lactose.

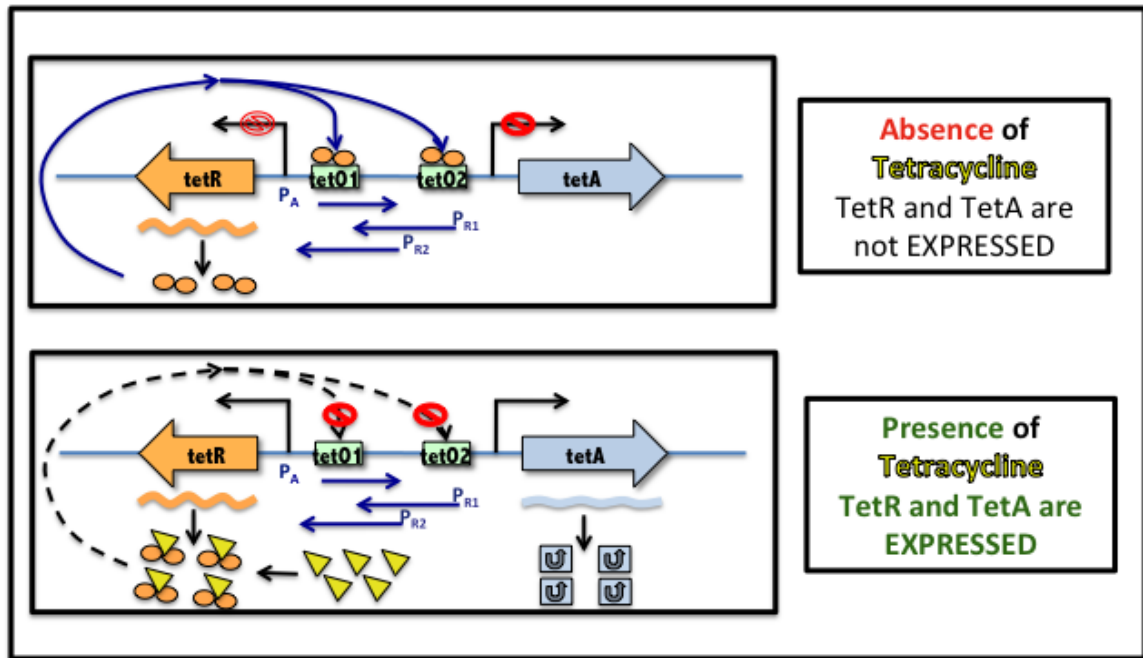


FIGURE 1.5. Genetic mechanism of the *tet* operon. In the absence of inducer (TCs), the TetR repressor binds onto operator regions (*tetO*₁ and *tetO*₂) to negatively control the expression of *tetA* and *tetR*. In the presence of inducer (TCs), TetR undergoes a conformational change so that it can no longer bind to the operators, thus allowing the expression of TetA required for the efflux of tetracycline.

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CHAPTER 2

DEVELOPMENT OF A REPRESSIBLE VACCINIA VIRUS EXPRESSION SYSTEM ASSEMBLED VIA A GENE CIRCUIT WITH NATURAL REPRESSORS FROM THE *LAC* AND *TET* OPERONS

2.1. ABSTRACT

A recombinant vaccinia virus (VACV) that contains elements from the lactose (*lac*) and the tetracycline (*tet*) operon systems of *E. coli* arranged in a gene circuit to conditionally control the expression of a reporter gene, enhanced green fluorescent protein (EGFP), was developed. In the absence of inducers, EGFP expression in infected cells was detectable, but less than a positive control virus expressing the gene constitutively under the same promoter. However, in the presence of increasing concentrations of a *lac* operon inducer alone (isopropyl- β -D-thiogalactoside, IPTG), EGFP expression increased in a dose dependent manner and at high IPTG concentrations, expression reached the same levels as a positive control virus. Additionally, in the presence of increasing concentrations of the *tet* operon inducer only (doxycycline, DOX), EGFP expression decreased in a dose dependent manner and at high concentrations of DOX, expression was repressed to the degree observed in a negative control virus that does not express EGFP. Thus, a dimmer-like modulation of expression can be achieved using different concentrations of either inducer. Furthermore, EGFP expression was modulated in the presence of both inducers, much like a double rheostat circuit, and even after infection. This modulatory expression system has potential applications for studying VACV genes in their life cycle or a means to regulate the expression of genes for the development of novel vaccines and cancer therapies.

2.2. INTRODUCTION

Vaccinia virus (VACV) belongs to the family *Poxviridae*, has a 190 kb dsDNA genome, and autonomously replicates in the host cell cytoplasm. Historically, VACV has gained much attention. First, it was used as a vaccine to eradicate variola virus, which caused smallpox disease (Fenner and World Health Organization, 1988). After this great achievement, advancements in genetic engineering allowed VACV to be used as a viral vector and to be studied in more detail (Mackett et al., 1982, Mackett et al., 1984, Moss, 1991, Moss, 1996). A popular technique used to study this complex virus is to place VACV genes under the conditional control of elements from either the tetracycline (*tet*) or the lactose (*lac*) operon from *E. coli* (Fuerst et al., 1989, Traktman et al., 2000), so that the role of the gene in the viral life cycle can be studied in the absence or presence of the respective operon inducers. In addition, these inducible expression systems can have practical applications to regulate the expression of VACV or heterologous genes, as there is a renewed interest for using VACV as a live viral vector for smallpox vaccines, heterologous vaccines, and cancer therapies (Verardi et al., 2012).

The construction of these inducer-dependent VACV recombinants entails an *E. coli* repressor gene (*lacI* or *tetR*) that is constitutively expressed by a VACV early/late promoter and an *E. coli* operator sequence (*lacO* or *tetO*) that is placed downstream from a VACV promoter and upstream from the start site of a VACV gene. In the absence of *lac* operon inducers, such as isopropyl- β -D-thiogalactoside (IPTG), or *tet* operon inducers, tetracyclines (TCs) such as tetracycline (TET) or doxycycline (DOX), the transcription of the gene of interest is blocked and the observed phenotypes (e.g., smaller plaque

phenotype or no mature virion formation via electron microscopy) can be interpreted to yield information on the essentiality and functionality of a gene of interest.

Although inducible systems are useful in characterizing VACV genes, only the induction of gene expression and the resulting phenotype can be effectively studied. After induction and infection, the removal of the inducer (e.g., by washing the cells with medium) is difficult, especially if expression at certain time points is being characterized. The inducer already present within the cell will not lead to full repression of gene expression in a timely fashion. Thus, a new modulatory system that allows for either quick repression or induction of gene expression by the addition (and not removal) of particular inducers offers a new and efficient way of studying the role of genes in the viral life cycle.

Despite the renewed interest in VACV as a replication competent vaccine and therapeutic vector, there is a growing subset of the population that is contraindicated against VACV vaccination as the infection could lead to severe adverse events, including death. This population includes the immunosuppressed, cancer therapy patients, individuals with atopic dermatitis, and individuals with heart conditions (Kemper et al., 2002). Thus, an approach to increase the safety of VACV vectors is to place a gene essential to viral replication under the control of a repressible system, which could serve as a built-in safety system. In cases where adverse events were to occur, transcription of a gene essential to viral replication could be blocked upon administration of an inducer, thus stopping the replication of the virus and most importantly, the progression of the adverse event.

In this study, elements from both the *lac* and the *tet* operons of *E. coli*, arranged via a gene circuit, were used to generate a recombinant VACV called vIRG, that is dependent on TCs to repress the expression of a reporter gene, enhanced green fluorescent protein (EGFP). Additionally, we found that in the presence of IPTG, EGFP expression can be induced. Thus, we describe a system that can modulate the EGFP expression much like a dimmer or double rheostats that can be controlled independently in the presence of appropriate inducers.

2.3. MATERIALS AND METHODS

Viruses and Cell Cultures. African green monkey kidney (BS-C-1) and human (HeLa S3) cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% TET tested fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) at 37°C in 5% CO₂. A clone derived from the Western Reserve (WR) strain of VACV (ATCC VR-2035) and all recombinant VACVs were propagated in BS-C-1 and HeLa S3 cells and titered by plaque assay in BS-C-1 cells.

Transfer Vectors. The construction of the vIRG transfer vector (pAT015) is detailed in Figure 2.1. Plasmid pAT009 is a designed synthetic plasmid (DNA2.0, Menlo Park, CA) that contains the *lacI* ORF, a multiple cloning site (MCS), and the late VACV P₁₁ promoter with a synthetic 22 bp *lacO* sequence (Sadler et al., 1983) located immediately downstream from the promoter transcriptional start site. The restriction sites *BglII* and *BspEI* flank the entire construct within pAT009 and this *BglII*-*BspEI* fragment was

cloned into the same sites of pSMART15 (Weber et al., 2007), generating pAT014. pSMART15 contains *tetR* under the control of a strong synthetic VACV early/late promoter ($P_{E/L}$) (Chakrabarti et al., 1997), the DsRed-Express gene under the control of an additional (back-to-back) VACV $P_{E/L}$ promoter with a 19 bp *tetO*₂ sequence placed directly downstream from the late transcriptional start site, and the *gpt-gus* fusion gene under the control of a synthetic early/late VACV P_{sel} promoter (Hammond et al., 1997), flanked by segments of the VACV thymidine kinase (TK) gene (left, TK_L and right, TK_R), which allow for homologous recombination with the VACV TK genomic region. The final transfer vector (pAT015) was generated by transferring the EGFP gene (Thastrup et al., 2001) from pAT010 (DNA2.0) into the *Bam*HI and *Eag*I sites of pAT014. Similarly, a transfer vector with EGFP under the control of P_{11} lacking the *tetO*₂ sequence was also generated to serve as a positive control. Additionally, transfer vectors with *lacI* under the control of $P_{E/L}$ with multiple operator sequences and arrangements were generated, with two tandem *tetO*₂ sequences separated by 2 bp ($2 \times tetO_2(2)$) or 11 bp ($2 \times tetO_2(11)$), as well as tandem *tetO*₁ and *tetO*₂ operators separated by 11 bp (*tetO*₁/*O*₂(11)) (Yao et al., 1998). These transfer vectors were generated by replacing the *Bgl*III-*Xma*I fragment of pAT015 (containing the $P_{E/L}$ -*tetO*₂ fragment) with the respective promoter fragments from plasmids pSMART16, pSMART17, and pSMART18 (Weber et al., 2007).

Generation of Recombinant VACVs. Recombinant VACVs vIRG ($1 \times tetO_2$), vIRG2 ($2 \times tetO_2(2)$), vIRG3 ($2 \times tetO_2(11)$), vIRG4 (*tetO*₁/*O*₂(11)), and vRGc (no *tetO*, positive control virus constitutively expressing EGFP) were generated via standard homologous recombination by transfection of the transfer vectors into BS-C-1 cell monolayers

infected with VACV strain WR (also used as the negative control) at a multiplicity of infection (MOI) of 0.05 plaque-forming units (PFU)/cell. Recombinant VACVs were plaque purified in selection medium (25 µg/ml mycophenolic acid, 250 µg/ml xanthine, 15 µg/ml hypoxanthine, in the absence or presence of 5 mM IPTG); plaques were visualized either with substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) or by selecting and collecting only EGFP⁺ expressing plaques detected via fluorescence microscopy. The purity of the recombinant VACVs (absence of parental virus) were confirmed by checking multiple dilutions of stocks in selection-free media (with 5 mM IPTG) for the presence of non-fluorescent (EGFP⁻) plaques. High titer stocks of each recombinant VACV were generated by infecting HeLa S3 cells at an MOI of 0.1. Infected cells were harvested 3 days post-infection (DPI) by centrifugation at 200 × g for 10 min. Cells were then lysed by freezing and thawing, sonicated, and trypsinized. Finally, cell lysates were clarified to remove contaminating cell debris by a second round of sonication and centrifugation at 200 × g for 5 min.

Determination of EGFP Expression. BS-C-1 cell monolayers in multiwell culture plates were infected with vIRG, vRGc, or WR (30 PFU or an MOI of 1) in the presence of medium only or various concentrations of IPTG, DOX, TET, or anhydrotetracycline (ATC). At 2 DPI, EGFP expression by isolated plaques or infected cells were determined by quantitative fluorescence microscopy (as described below). In certain instances, cell monolayer, were washed with 1× phosphate-buffered saline (PBS) pH 7.2, resuspended in equal parts of 1× PBS and 2% neutral buffered formalin (1% final concentration), and analyzed with a fluorescence plate reader (as described below) within 24 hr.

Modulation of EGFP Expression. BS-C-1 cell monolayers in multiwell culture plates were infected with vIRG, vRGc, or WR (30 PFU or an MOI of 1) in the presence of medium only or various concentrations of DOX and IPTG, and 2 DPI plaques were imaged via fluorescence microscopy. Additionally, HeLa S3 cell monolayers were infected at an MOI of 1 in various concentrations of DOX or IPTG, and 24 hr later cells were trypsinized for 5 min on plate shaker (low setting), centrifuged $300 \times g$ for 10 min, resuspended in equal parts of $1 \times$ PBS and 2% neutral buffered formalin (1% final concentration), and analyzed by flow cytometry within 24 hr. Briefly, 10,000 cells were analyzed in a FACScan (Becton Dickinson, Rutherford, NJ) flow cytometer and the CellQuest software (Becton Dickinson) was used to gate cell populations based on forward and side scatter and to determine the mean fluorescence (488 nm excitation and 530/30 emission).

EGFP Expression Kinetics. First, the inducibility of EGFP expression was tested by infecting BS-C-1 cell monolayers with vIRG, vRGc, or WR at an MOI of 1 in the presence of 1 mM IPTG added at the time of infection or several times post-infection. Cells were imaged and quantified at each time point as described below. In addition, the repressibility of EGFP expression was tested by first infecting cells as above in the presence of 1 mM IPTG, and then adding DOX (100 ng/ml) at various time points post-infection.

Imaging and Image Quantification. Infected cells and plaques were imaged using an inverted fluorescence microscope (Axio Observer D1, Carl Zeiss, Thornwood, NY) with or without a green bandpass filter (XF100-2, Omega Optical, Brattleboro, VT). EGFP expression levels were quantified with the AxioVision software, release 4.8.1 (Carl Zeiss).

Fluorescence Quantification with a Microplate Reader. The fluorescence readings of infected cells were obtained with a fluorescence plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek Instruments, Winooski, VT) using a 485/20 nm excitation and a 528/20 nm emission filter pair. EGFP expression levels are quantified with the KC4 v3.4 software (BioTek Instruments).

2.4. RESULTS

EGFP Expression Levels by vIRG in the Absence of *lac* and *tet* Operon Inducers is Lower than Expected. In the absence of inducers, BS-C-1 cells infected with vIRG, a negative control virus (WR), or a positive control virus (vRGc) all displayed the expected cytopathic effect (CPE) 2 DPI and when observed under brightfield microscopy (Figure 2.2). Under fluorescence microscopy, vIRG infected cells displayed detectable levels of EGFP expression (Figure 2.2). However, the observed EGFP expression levels were significantly lower than those observed for the positive control virus (vRGc) (Figure 2.2). This result was unexpected as we anticipated that vIRG would be completely unrepressed in the absence of inducers.

IPTG Induces Higher EGFP Expression Levels by vIRG in a Dose Response

Manner. A likely mechanism for the observed intermediate level of EGFP expression by vIRG is the leaky expression of *lacI* under the control of the strong early/late synthetic promoter $P_{E/L}$. In such case, any expressed LacI repressor would bind to the *tetO*₂ sequence downstream from the EGFP promoter, reducing EGFP expression. To test this possibility, BS-C-1 cells were infected with vIRG in the presence of 1 or 5 mM of IPTG, a *lac* operon inducer. Previously, concentrations of IPTG between 0.1-5 mM were used in a *lac* inducible expression system in VACV (Fuerst et al., 1989). The CPE observed by brightfield microscopy 2 DPI in cells treated with 5 mM IPTG was indistinguishable from the CPE observed in cells that did not receive IPTG (Figure 2.3). Moreover, fluorescence microscopy indicated that infected cells treated with IPTG displayed significantly higher levels of EGFP expression levels when compared with untreated cells (Figure 2.3). To quantitate the levels of EGFP expression, cells were infected at an MOI of 1 in the presence of 1 mM IPTG (Figure 2.4) and analyzed by quantitative fluorescence microscopy (Figure 2.5). The addition of IPTG resulted in EGFP expression levels by vIRG that were at the same levels as vRGc. This would be expected since any potential LacI repressor present (e.g., by leaky expression) should undergo a conformational change in the presence of IPTG so that it no longer binds onto its cognate *lacO* sequence. Furthermore, when increasing concentrations of IPTG were added to cells infected with vIRG, EGFP expression levels increased in a dose dependent manner (Figure 2.6). Therefore, in a system in which basal EGFP expression levels were intermediate, the addition of IPTG resulted in an inducible expression system.

DOX Completely Represses EGFP Expression by vIRG in a Dose Response Manner.

Although we were able to demonstrate that EGFP expression was inducible, our initial goal was to develop a system that was able to represses EGFP expression in the presence of *tet* operon inducers. Previously, a variety of TET derivatives have been used in *tet* systems. For example, TET and DOX at concentrations between 0.1-1 $\mu\text{g/ml}$ were successfully used in the inducible VACV expression systems (Traktman et al., 2000, Weber et al., 2007). In addition, ATC (a TET derivative with minimal antibiotic activity) has been used at concentrations of 0.4 μM (185 ng/ml) in bacterial expression systems (Scholz et al., 2003, Scholz et al., 2004). Thus, to determine if EGFP expression was repressible, DOX, TET, and ATC were added to cells infected with vIRG and expression of EGFP was quantified with a fluorescence microplate reader (Figure 2.7). At 2 DPI, no detectable differences in CPE were observed between any TC-treated and untreated cells via brightfield microscopy (data not shown). In the presence of TCs, EGFP expression was repressed to differing degrees depending on the type and concentration of TC used. The repression of EGFP expression was very sensitive to DOX, followed by ATC and TET. DOX at 1 ng/ml was sufficient to repress EGFP expression to the level of the negative control virus, WR. At higher concentrations of ATC, there was a small detectable increase in fluorescence, which was also confirmed by fluorescence microscopy (data not shown). Additionally, ATC is associated with side effects such as cutaneous phototoxicity and Fanconi-type syndrome in humans (Burgos et al., 2011). Therefore, DOX was used for further characterization, as it is the most sensitive in repressing EGFP expression, it does not exhibit a high degree of autofluorescence, and is an FDA approved drug.

To demonstrate that EGFP expression levels can be repressed to fluorescence levels compared to WR, cells were infected with WR or vIRG in the absence or presence of 100 ng/ml of DOX (Figure 2.8). In the absence of DOX, EGFP expression (measured in arbitrary fluorescence units) by vIRG was higher than in WR-infected cells. In the presence of DOX, EGFP expression by vIRG was repressed to the same level as WR. WR fluorescence levels stayed the same in the absence or presence of DOX. Finally, as increasing concentrations of DOX were added to cells infected with vIRG, EGFP expression levels decreased in a dose dependent manner and at 10 ng/ml, EGFP expression was repressed to levels comparable to WR (Figure 2.9). Thus, we were able to show that this novel VACV expression system has the ability to display both inducible and repressible gene expression phenotypes in the presence of the appropriate inducers.

Rheostat Modulation of EGFP Expression. The inducible and repressible capabilities of vIRG allow the modulation of EGFP expression in a dimmer-like fashion, using the appropriate inducers. To demonstrate the range of EGFP expression in this system, cells were infected with vIRG in the absence or presence of increasing concentrations of DOX or IPTG. As observed initially, cells infected with vIRG in the absence of inducers (media only) yielded intermediate levels of EGFP expression (Figure 2.10). EGFP expression could be repressed by treating with increasing concentrations of DOX, or could be induced by adding increasing concentrations of IPTG.

Flow cytometry (a more sensitive tool to detect EGFP expression) was also used to quantify the range of gene expression that can be achieved with vIRG. In a preliminary study, HeLa S3 cells were infected with vIRG, WR or vRGc in the absence

or presence of varying concentrations DOX or IPTG (Figure 2.11). vIRG exhibited the same trends of repression and induction as observed by fluorescence microscopy and microplate reader. However, EGFP expression did not seem to be fully repressed to WR levels or induced to the same levels of vRGc. These results could be due to a variety of factors such the use of a different cell line, small number of cells analyzed, acquisition or analysis settings (such as the gating of infected cells, since at an MOI of 1 only about 63% of the cells are expected to be infected), and the fact that control virus measurements were taken in the absence of inducers. Nonetheless, both methods show that EGFP expression by vIRG could be modulated in the presence of increasing concentrations of DOX or IPTG.

Next, we tested the system in BS-C-1 cells infected with vIRG in the presence of varying concentrations of both DOX and IPTG (Figure 2.12). EGFP expression in these conditions followed the same trends of induction in the presence of increasing concentrations of IPTG and repression in the presence of increasing concentrations of DOX. Furthermore, in the presence of the highest concentration of DOX (where EGFP expression is similar to the level of the negative controls virus), the addition of increasing concentrations of IPTG to the induction of EGFP expression, without the removal of DOX. Conversely, in the presence of the highest concentration of IPTG (that induces EGFP expression to maximal levels), the addition of DOX leads to the repression of EGFP expression. Thus, the vIRG system also allows a double rheostat-like modulation of gene expression, so that two independent “switches” (inducers) can be used to simultaneously modulate EGFP expression.

Kinetics of EGFP Induction by vIRG. To study the kinetics of EGFP expression by vIRG, BS-C-1 cells were infected with vIRG, WR, or vRGc and treated with IPTG (1 mM) at various time points post-infection and EGFP expression was quantified at every time point (Figure 2.13). The different IPTG treatments did not alter the EGFP expression kinetics for WR and vRGc. As observed initially, in the absence of IPTG, vIRG expressed intermediate levels of EGFP (fluorescence measurements are higher than WR). EGFP expression by vIRG treated with IPTG at 0, 3 and 12 hr did not differ significantly and peaked at 48 hr post-infection (similar to vRGc). This is most likely due to the late promoter (P_{11}) that drives EGFP expression in this system. Conversely, EGFP expression by vIRG increased dramatically after the addition of IPTG between 18 and 48 hr post-infection. Thus, induction of this system can be tightly regulated even after infection.

Kinetics of EGFP Repression by vIRG. To study the kinetics of repression of EGFP expression, BS-C-1 cells were infected with vIRG in the presence of 1 mM IPTG (to induce expression) and then treated with DOX (100 ng/ml) at several time points post-infection (to repress expression) (Figure 2.14). As observed initially, vIRG grown in medium only had measurable levels of EGFP expression (fluorescence higher than WR). In the presence of IPTG only, vIRG expressed levels of EGFP comparable to the positive control virus (vRGc). In the presence of IPTG, addition of DOX between 0-12 hr post-infection produced expression curves that were similar to vIRG grown in the absence of

DOX. Thus, DOX treatment between 0 and 12 hr post-infection halts the expression of EGFP. Again, this is likely due to the late P_{11} promoter that drives expression of EGFP to high levels only after 12 hr post-infection, as observed in vRGc. Conversely, cells infected with vIRG in the presence of IPTG and treated with DOX at 18 and 24 hr displayed a significant increase in EGFP expression before DOX exposure; however, EGFP expression was repressed and eventually plateaued after DOX exposure. Thus, repression in this VACV gene expression system can be tightly regulated even after infection.

Addition of *tetO* Operators in Different Numbers and Arrangements Does Not

Prevent Leaky Expression of LacI.

A number of recombinant VACVs derived from vIRG were developed with the *lacI* gene under the control of the $P_{E/L}$ promoter with multiple *tetO* operator sequences and arrangements: two tandem *tetO*₂ sequences separated by 2 bp (2×*tetO*₂(2), vIRG2) or 11 bp (2×*tetO*₂(11), vIRG3), as well as tandem *tetO*₁ and *tetO*₂ operators separated by 11 bp (*tetO*₁/*O*₂(11), vIRG4), as described by Weber et al. (Weber et al., 2007). BS-C-1 cells were infected with the different viruses in the absence or presence of 5 mM IPTG, and isolated plaques were imaged by fluorescence microscopy (Figure 2.15). The levels of EGFP expression in the absence of IPTG do not differ between the different recombinant VACVs, indicating that the different number and arrangements of *tetO* operators tested were not able to stop leaky expression of LacI.

2.5. DISCUSSION AND CONCLUSIONS

We developed a gene modulation system in VACV that is not only repressible, but also inducible in the presence of the appropriate inducers. However, it was not our initial expectation that vIRG would also result in an inducible system as the development of this project initially focused on the design of a repressible system. The vIRG expression system is able to exhibit both inducible and repressible capabilities likely due to the unexpected leaky expression of *lacI*. Therefore, in an attempt to control *lacI* leaky expression, new transfer vectors were designed with the addition of tandem *tetO* operator sequences (in different arrangements) placed downstream from the P_{E/L} promoter controlling *lacI* expression. However, preliminary studies indicate that this strategy does not seem to lower the leaky expression of *lacI* significantly. This is likely because repression of early VACV promoters using repressor proteins such as LacI and TetR does not seem to be achievable using this technology, as evidenced by recent unpublished data from the Verardi Lab (Weber, Verardi et al, unpublished data). Thus, an alternative method to control this potential leaky expression of *lacI* is to change the current *lacI* promoter (the early/late P_{E/L} promoter) to the late P₁₁ promoter, which has been successfully used to tightly control gene expression in VACV (C. Hagen, unpublished data).

Based on this theory, we can outline a potential gene circuit mechanism underlying the control of gene expression in vIRG. The constitutive expression of *tetR* should provide a continuous supply of the TetR repressor protein (Figure 2.16). In the absence of inducers, there is probably an expression timing issue that leads to intermediate levels of EGFP expression. We know that early transcription occurs soon

after infection (virus entry). If *tetR* and *lacI* are under the control of the same strong $P_{E/L}$ promoter, then both *lacI* and *tetR* expression will ensue at rates that are initially the same. Thus, immediately after infection, *lacI* is being expressed, albeit at low levels. As the TetR protein accumulates, then it can finally bind to *tetO* operator sequences to halt further transcription of *lacI*. However, since the reporter gene (EGFP) is under a late promoter, any LacI already present in the cell will interfere with its expression, and hence the intermediate levels of expression.

In conclusion, we have demonstrated a novel modulatory expression system in VACV that is sensitive to both the *lac* and the *tet* operon inducers, is inducible as well as repressible, is dose-dependent, and allows temporal regulation of gene expression even after infection and without the removal of the previous inducer. This novel expression system has an array of potential applications in basic virology (e.g., the study of VACV genes and their function or temporal essentiality) and translational animal and human medicine (e.g., safer vaccines and therapeutic viral vectors). One application would be to regulate a VACV gene of interest using this system and determine the amount and timing of expression necessary to allow morphogenesis and replication, so that DOX and IPTG could be used like independent rheostats controlling gene expression. In another application, a gene that is known to be essential for viral replication could be placed under the control of this system to serve as a potential safety mechanism for VACV vectors used for vaccination or cancer therapy. If VACV-associated complications were to arise, DOX administration would lead to the repression of the gene, stopping viral replication and halting the adverse event.

2.6. FIGURES

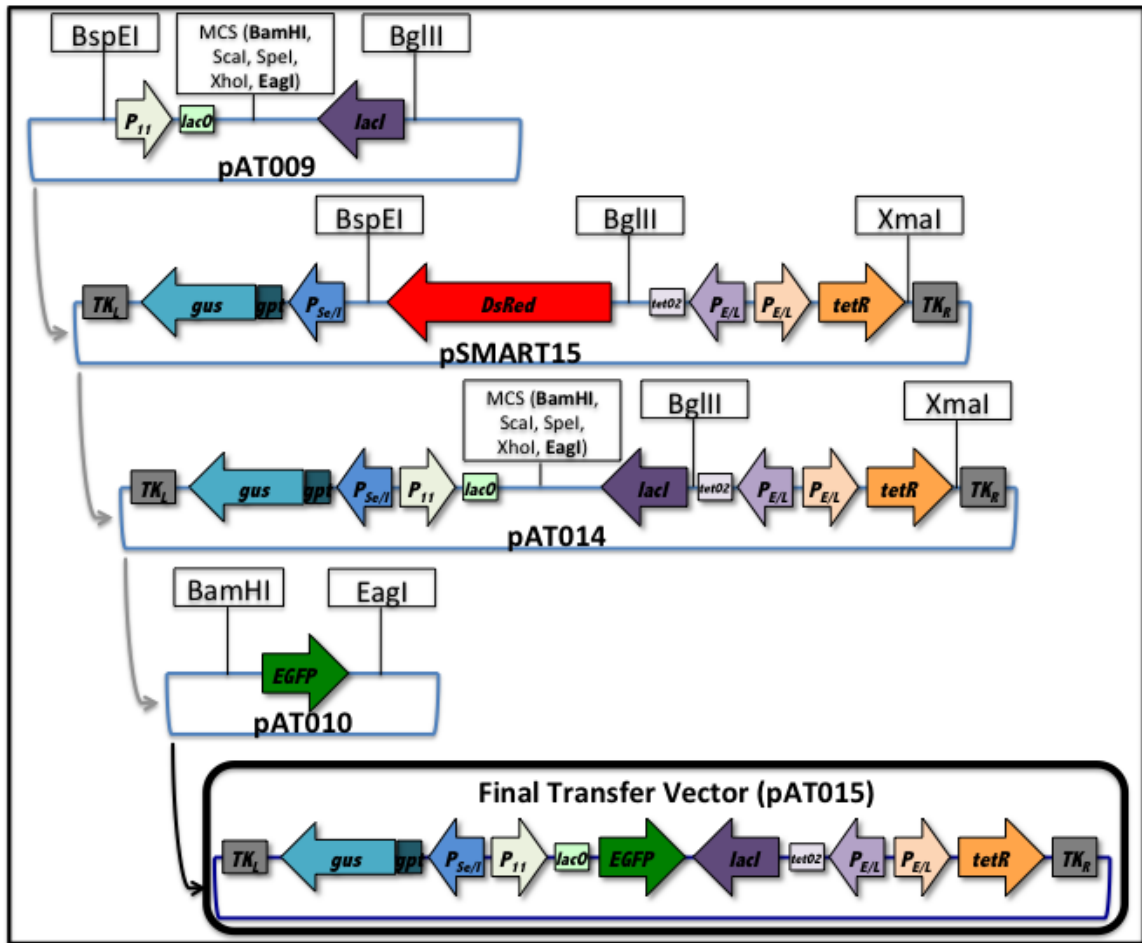


FIGURE 2.1. Construction of the transfer vector pAT015 for the generation of vIRG. The cloning steps leading to the generation of the final transfer vector are shown. pAT015 contains the *gpt-gus* fusion gene under the control of an early/late VACV promoter ($P_{Se/L}$), the EGFP gene under the control of an engineered P_{11} promoter with a *lacO* sequence located directly downstream, the *tetR* gene under the control of a constitutive strong early/late promoter ($P_{E/L}$), and the *lacI* gene under the control of an additional (back-to-back) $P_{E/L}$ promoter with a *tetO₂* sequence located directly downstream, all flanked by TK_L and TK_R sequences that direct recombination with the TK gene of VACV.

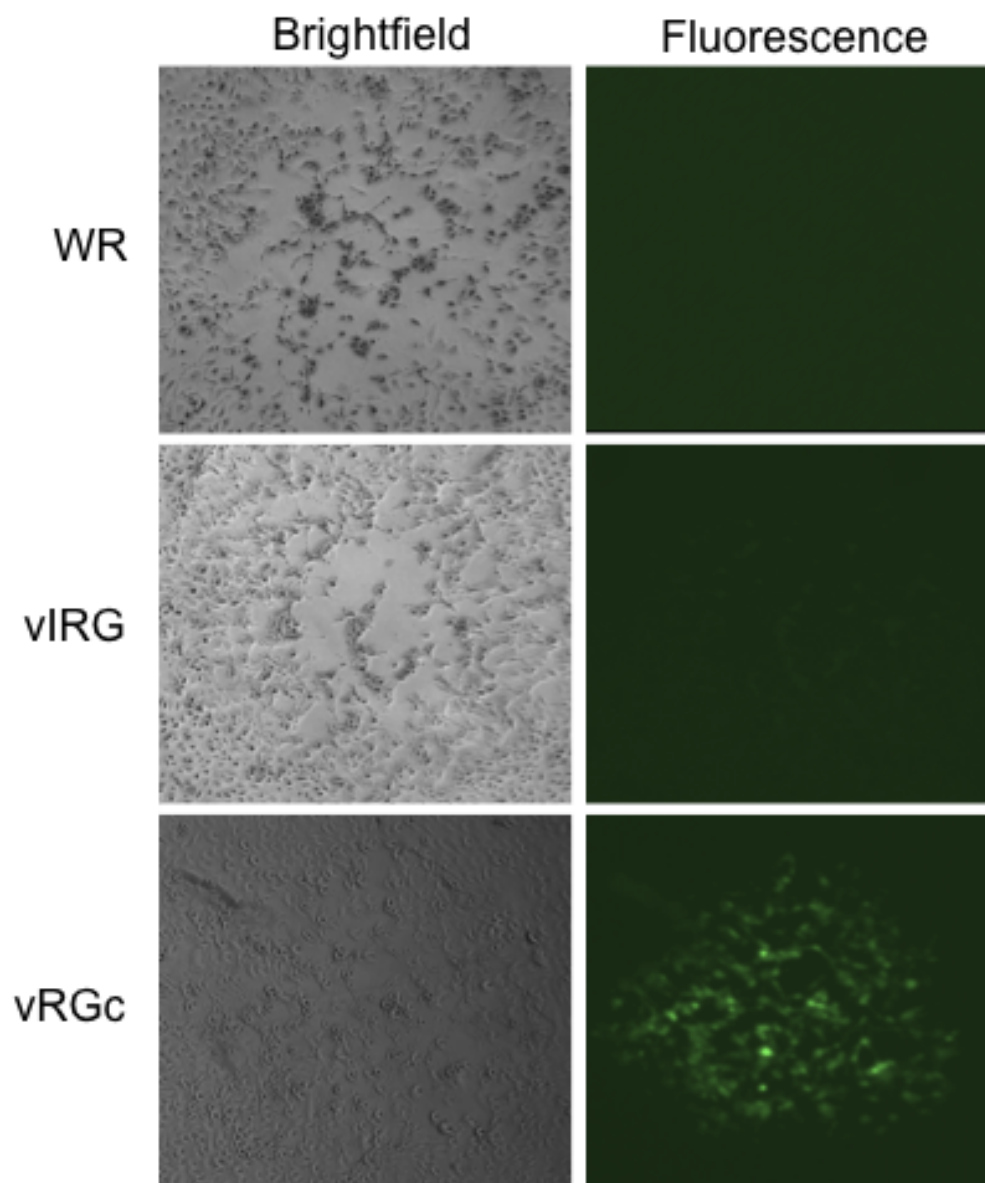


FIGURE 2.2. EGFP expression levels in the absence of *lac* and *tet* operon inducers.

BS-C-1 cells infected with WR, vIRG, or vRGc in the absence of inducers displayed typical CPE 2 DPI, and single isolated plaques were imaged by brightfield and fluorescence microscopy. vIRG-infected cells displayed significantly lower fluorescence levels when compared to vRGc (positive control virus), but significantly higher fluorescence levels when compared to WR (negative control virus).

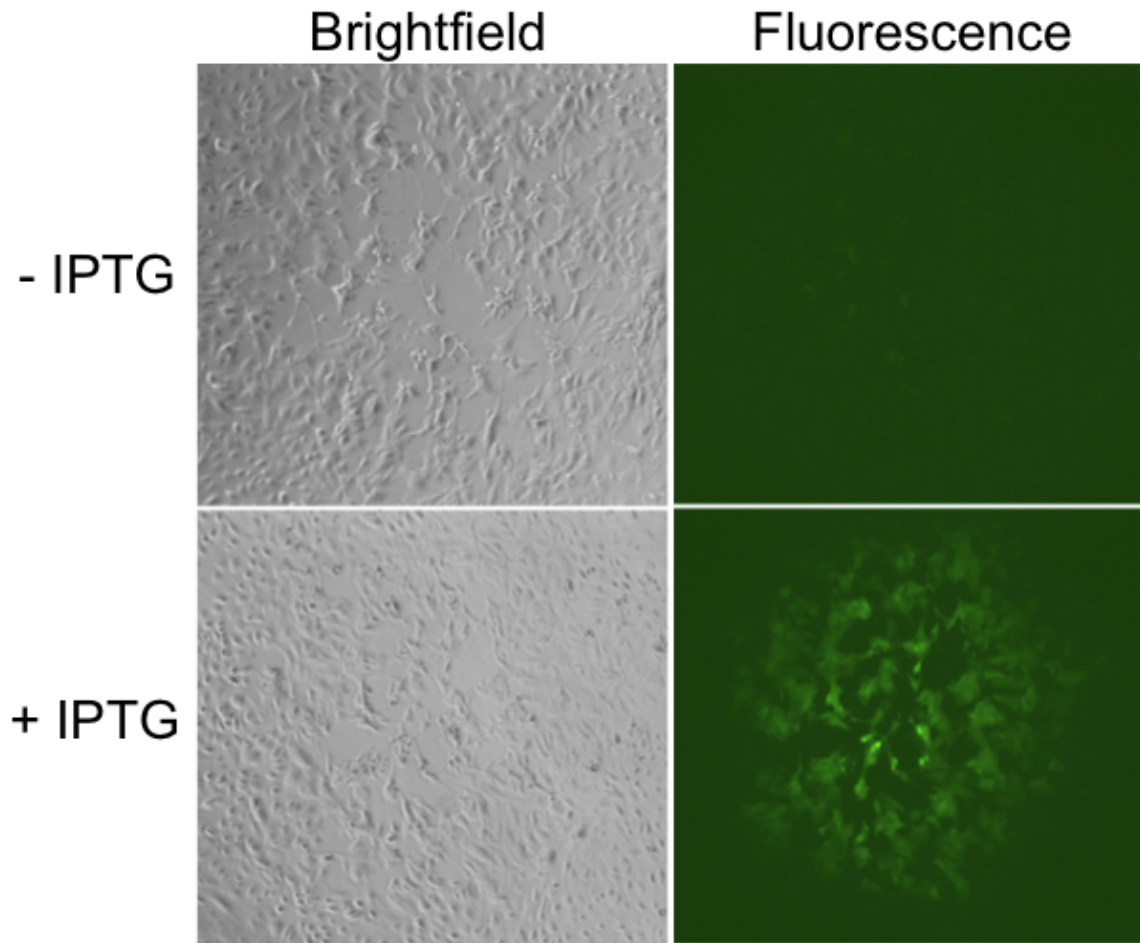


FIGURE 2.3. Cells infected with vIRG in the presence of 1 mM IPTG express higher levels of EGFP. BS-C-1 cells were infected with vIRG in the absence (- IPTG) or presence (+ IPTG) of 5 mM IPTG and imaged 2 DPI. All infected cells displayed similar CPE and single isolated plaques were imaged by brightfield and fluorescence microscopy. Cells infected with vIRG in the presence of IPTG displayed high levels of fluorescence when compared to infected cells not treated with IPTG.

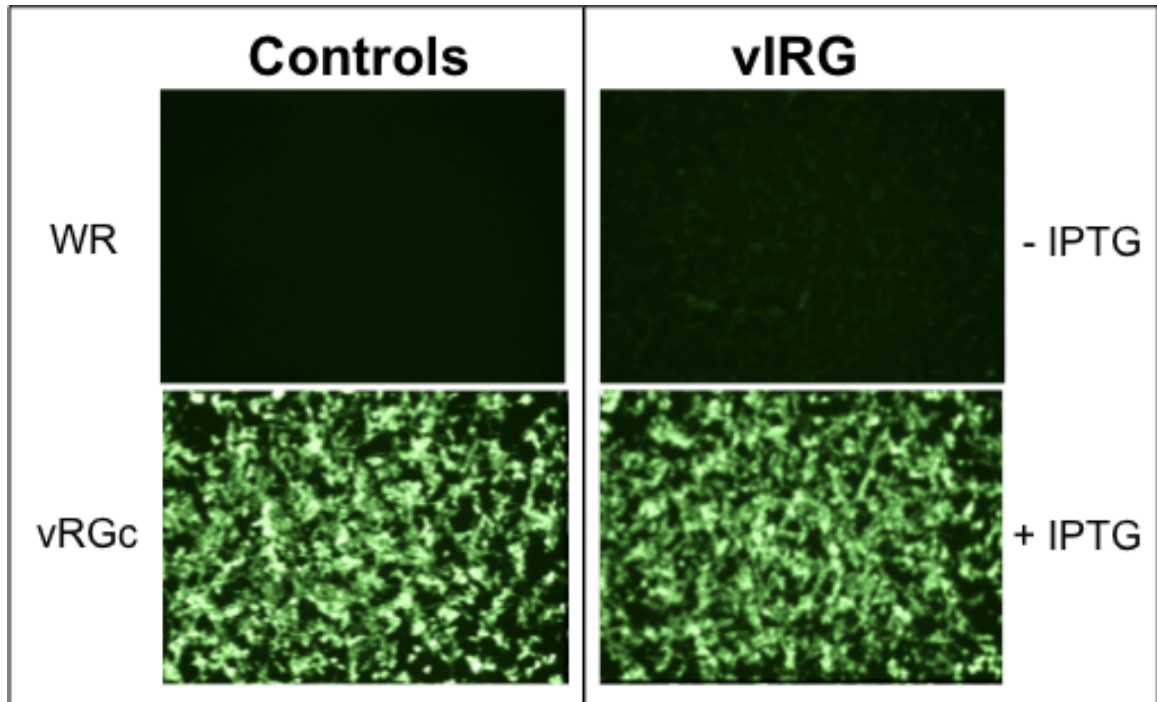


FIGURE 2.4. EGFP expression levels in vIRG-infected cells treated with IPTG are high. BS-C-1 cells were infected at an MOI of 1 with WR, vRGc, or vIRG in the absence (- IPTG) or presence (+ IPTG) of 1 mM IPTG, and 2 DPI cells were imaged by fluorescence microscopy. The EGFP expression levels detected in vIRG-infected cells treated with IPTG were higher than in the absence of IPTG.

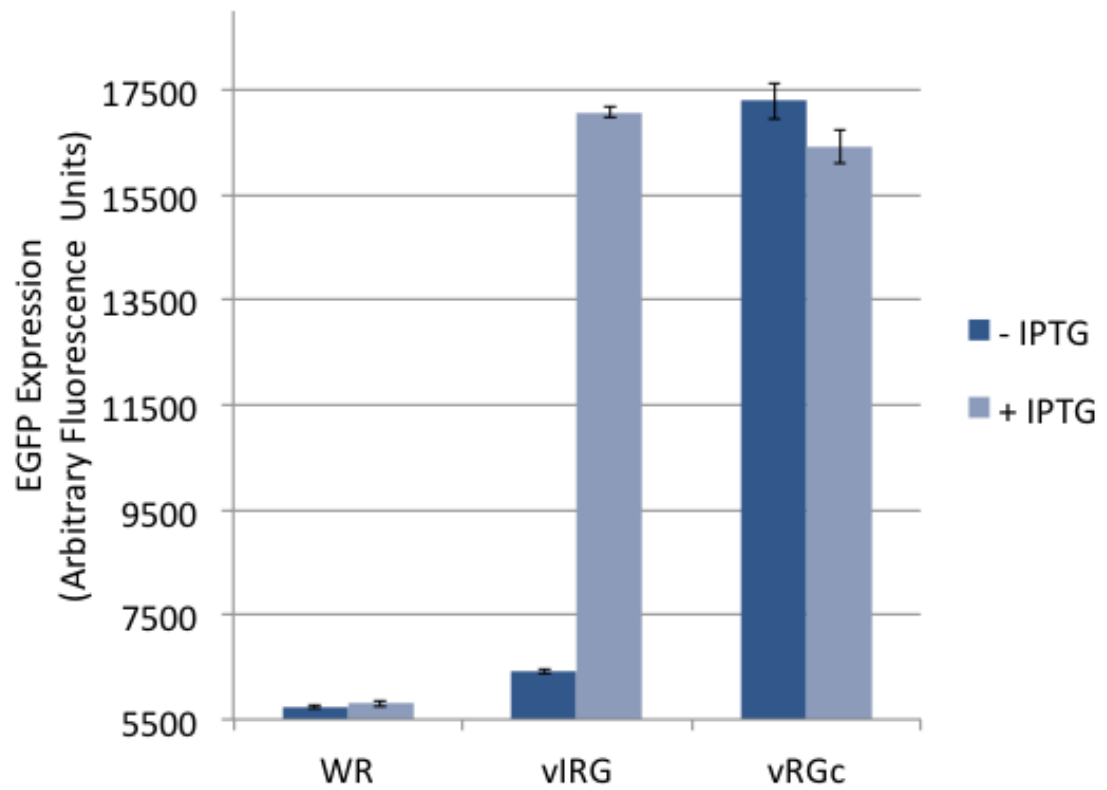


FIGURE 2.5. Quantification of EGFP expression in BS-C-1 cells infected with vIRG in the absence or presence of IPTG. BS-C-1 cells were infected at an MOI of 1 with WR, vRGc, or vIRG in the absence (- IPTG) or presence (+ IPTG) of 1 mM IPTG, and 2 DPI cells were imaged by fluorescence microscopy and EGFP expression was quantified. The EGFP expression levels detected in vRGc and vIRG-infected cells treated with IPTG were indistinguishable and higher than vIRG-infected cells in the absence of IPTG. Error bars are SEM, n=3.

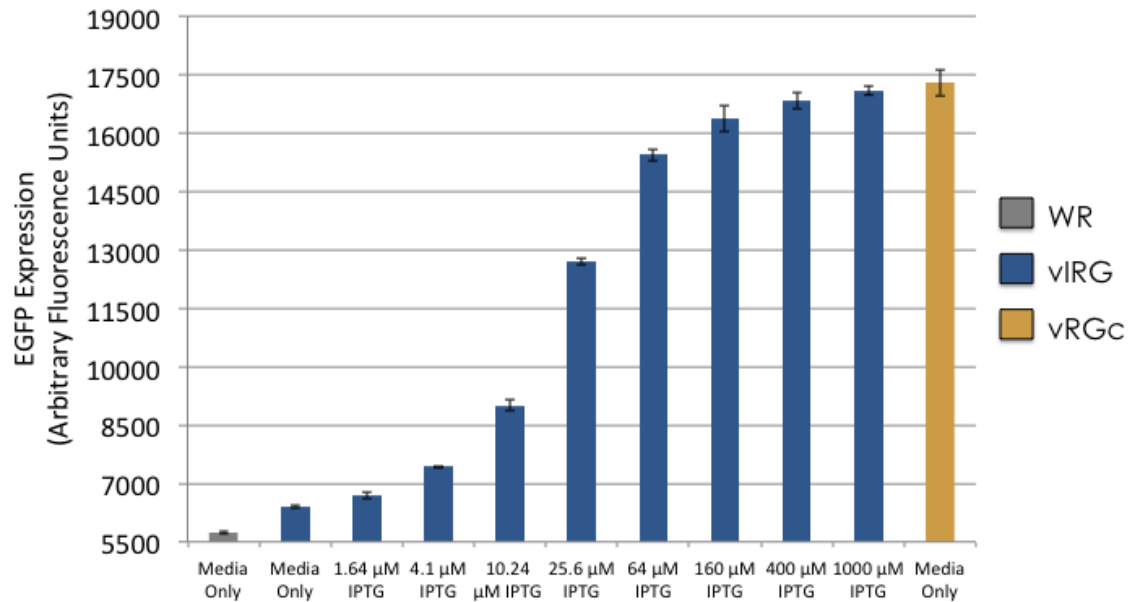


FIGURE 2.6. IPTG induces EGFP expression by vIRG in a dose response manner.

BS-C-1 cells were infected at an MOI of 1 with WR, vIRG, or vRGc in the absence or presence of increasing concentrations of IPTG, and 2 DPI cells were imaged by fluorescence microscopy and EGFP expression was quantified. EGFP expression by vIRG followed a dose-response curve. Error bars are SEM, n=3.

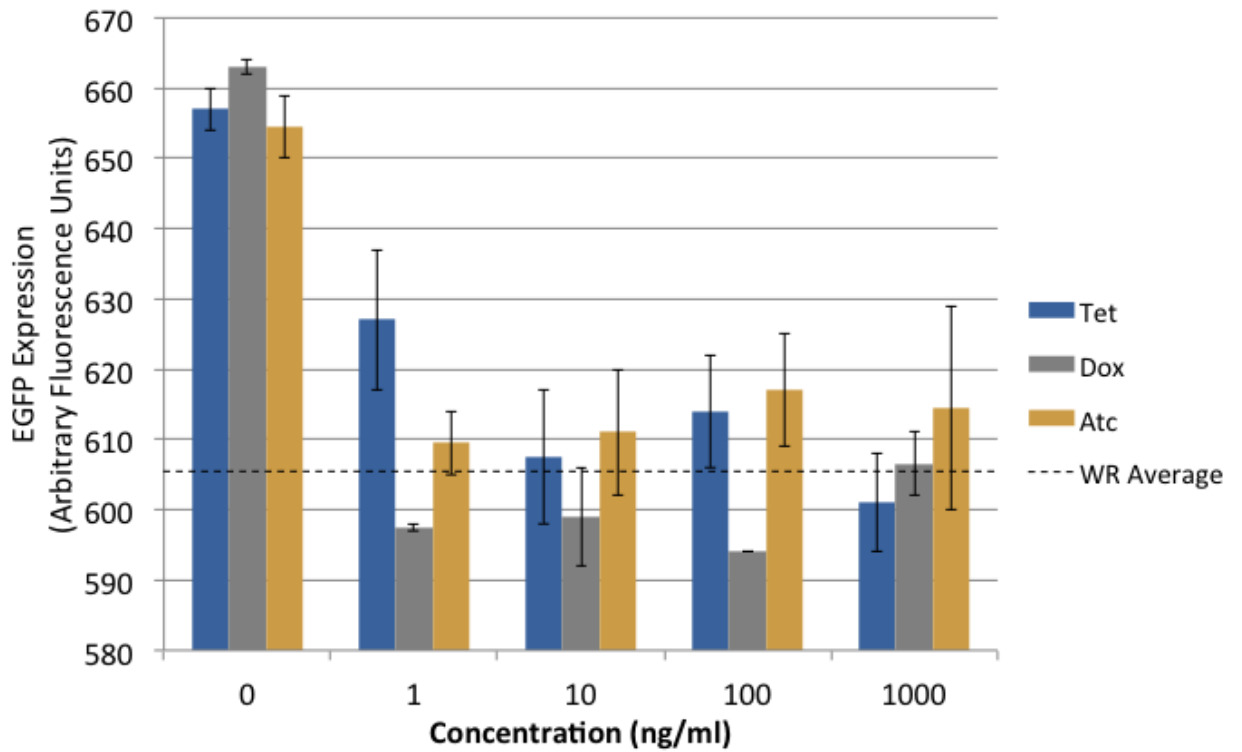


FIGURE 2.7. EGFP expression by vIRG is repressible by TCs. BS-C-1 cells were infected at an MOI of 1 with vIRG or WR in the absence or presence of varying concentrations of TET, DOX, or ATC, and 2 DPI EGFP expression was quantified with a microplate reader. EGFP expression levels were repressed in the presence of all TCs, with DOX exhibiting the best repressible profile. Error bars are SEM, n=2.

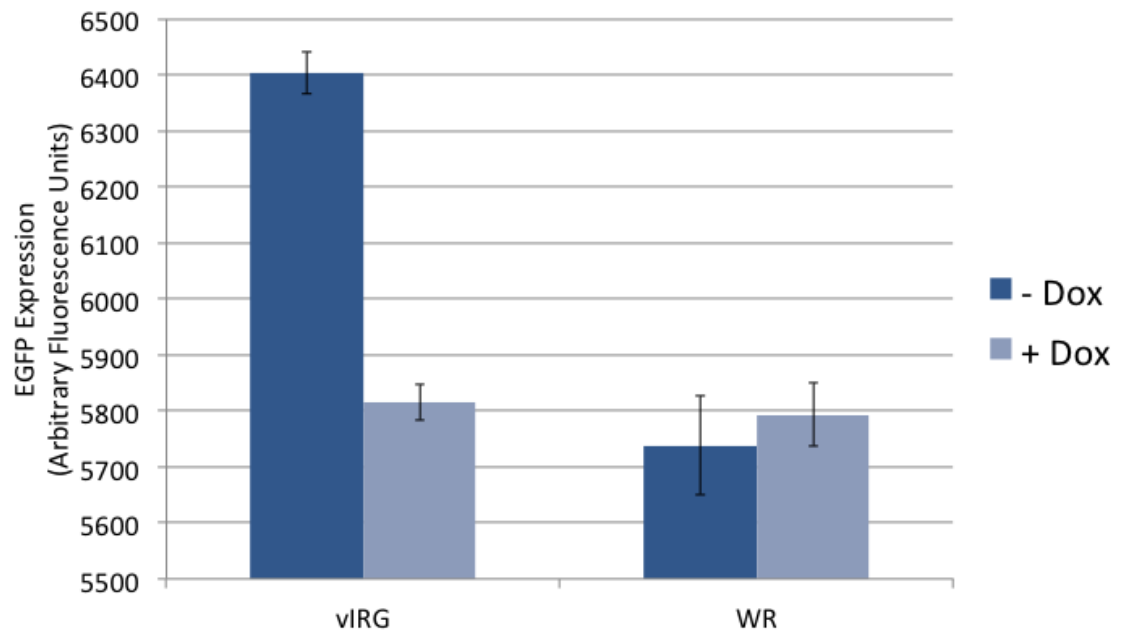


FIGURE 2.8. EGFP expression by vIRG is completely repressed in the presence of DOX. BS-C-1 cells were infected at an MOI of 1 with WR or vIRG in the absence (- DOX) or presence (+ DOX) of 100 ng/ml DOX, and 2 DPI cells were imaged by fluorescence microscopy and EGFP expression was quantified. EGFP expression by vIRG in the presence of DOX was repressed to levels comparable to WR. Error bars are SEM, n=3.

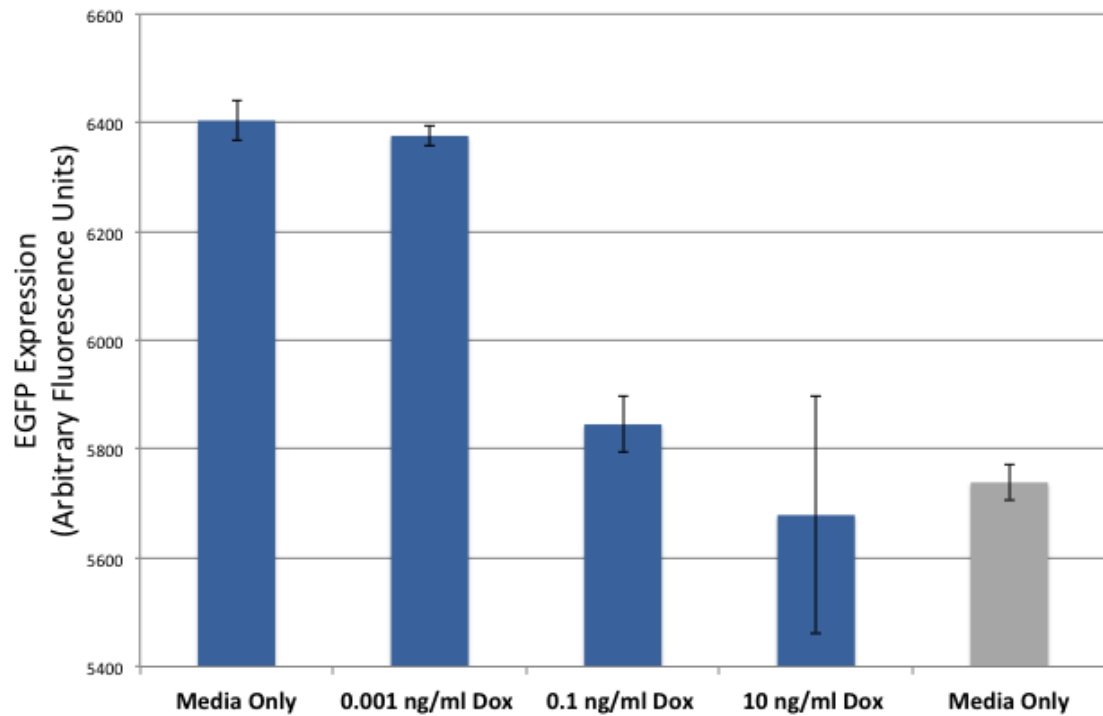


FIGURE 2.9. Repressible dose response to increasing concentrations of DOX. BS-C-1 cells were infected at an MOI of 1 with WR or vIRG in the absence or presence of increasing concentrations of DOX, and 2 DPI cells were imaged by fluorescence microscopy and EGFP expression was quantified. Error bars are SEM, n=3.

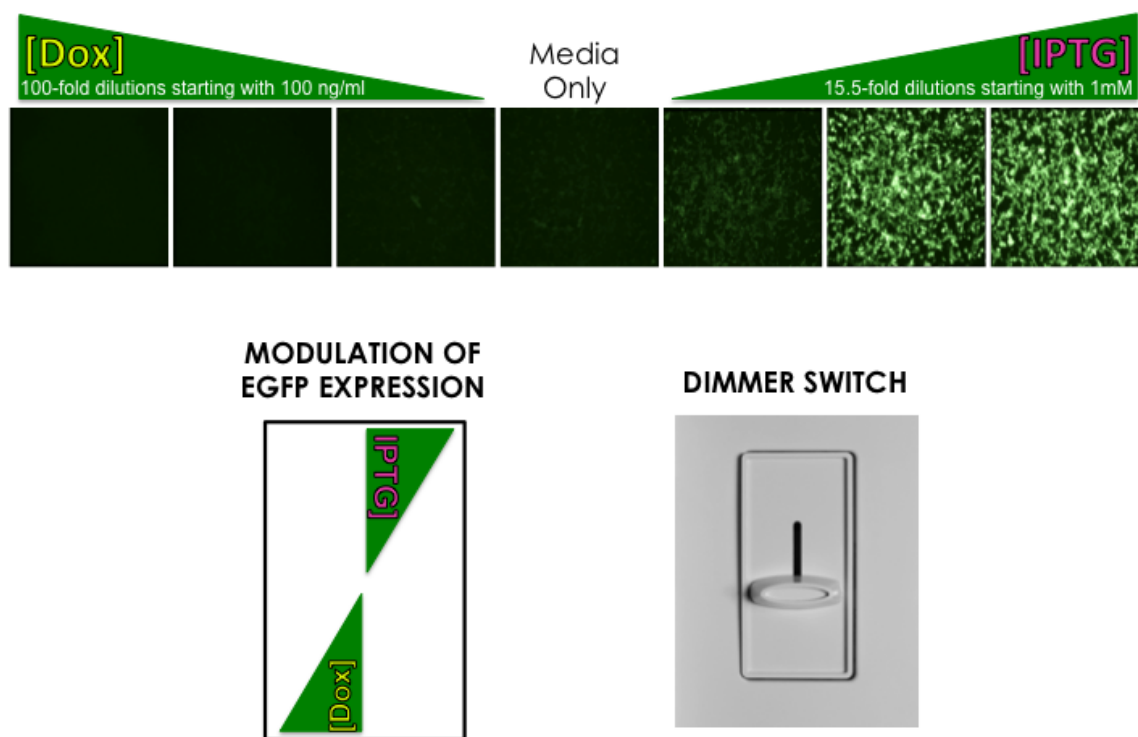


FIGURE 2.10. Dimmer-like modulation of EGFP expression in vIRG as determined by fluorescence microscopy. BS-C-1 cells were infected at an MOI of 1 with vIRG in the absence or presence of varying concentrations of DOX or IPTG, and 2 DPI cells were imaged by fluorescence microscopy. EGFP expression levels vary from levels comparable to a negative control virus (WR) in the presence of DOX to levels comparable to a positive control virus (vRGc) in the presence of IPTG.

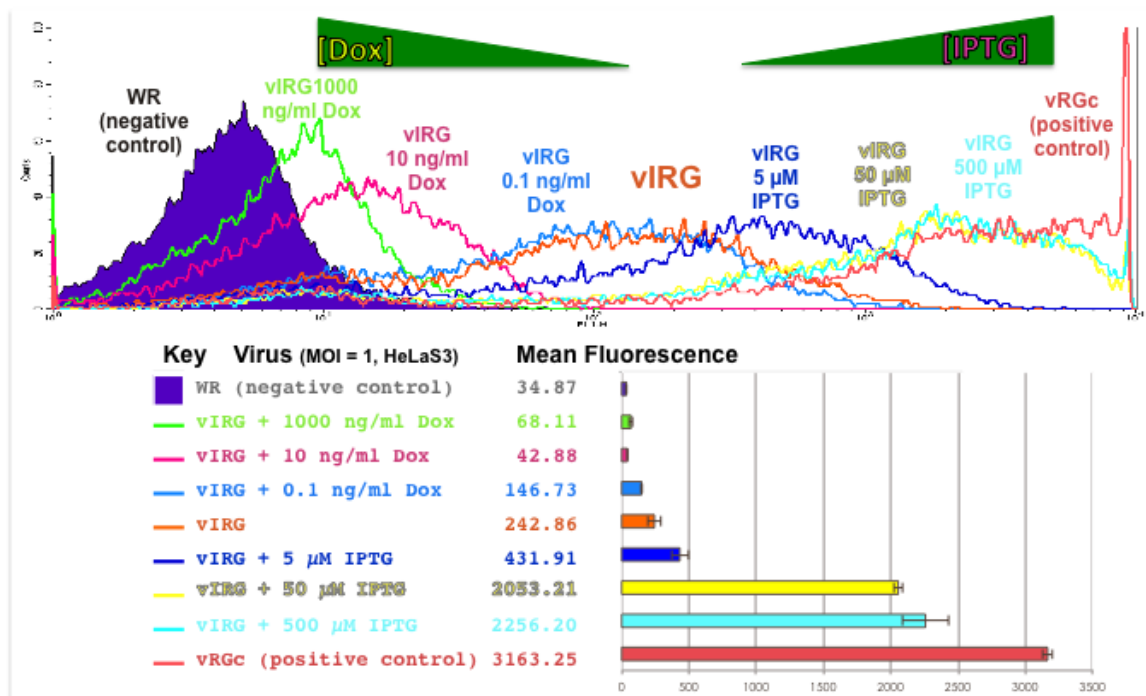


FIGURE 2.11. Dimmer-like modulation of EGFP expression in vIRG as determined by flow cytometry. HeLa S3 cells were infected at an MOI of 1 with vIRG, WR, or vRGc in the absence or presence of varying concentrations of DOX or IPTG. Cells were analyzed by flow cytometry (histograms in upper panel) and mean fluorescence values were determined (lower panel). EGFP expression by vIRG can be modulated much like a dimmer. Error bars are SEM, n=3.

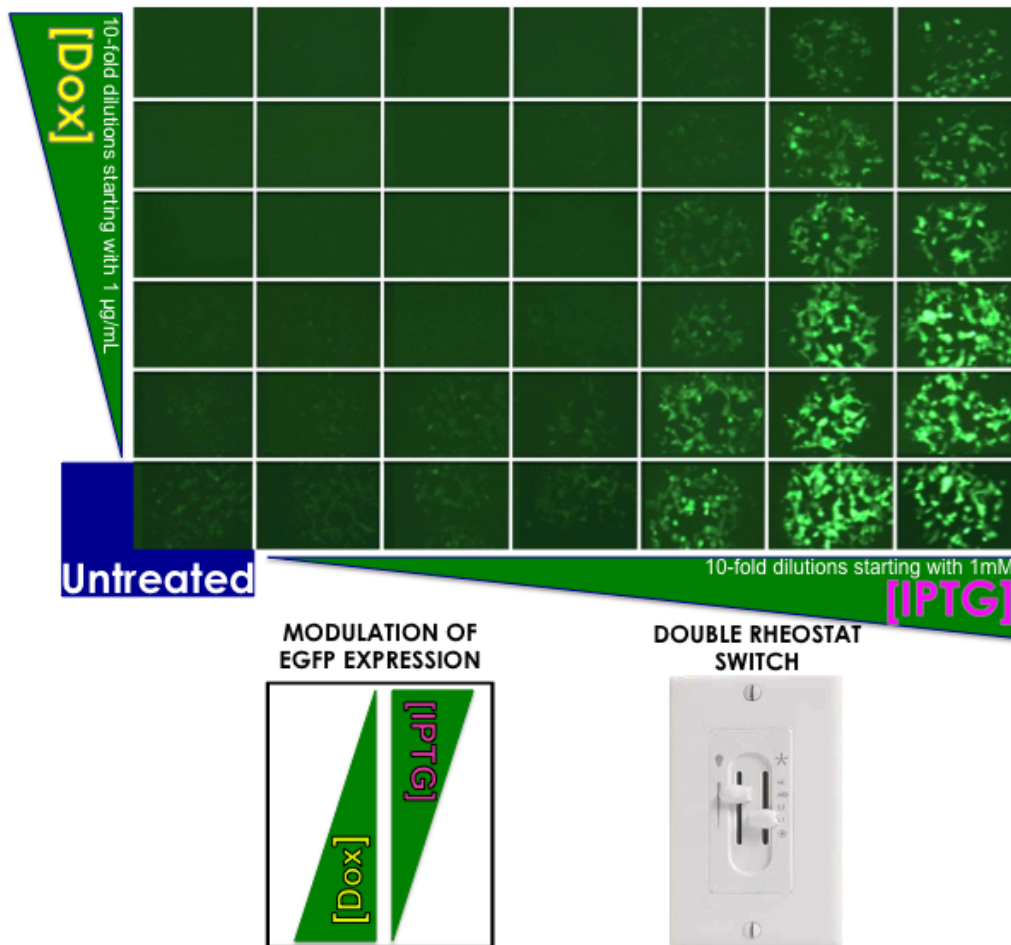


FIGURE 2.12. Double rheostat-like modulation of EGFP expression with IPTG and DOX. BS-C-1 cells were infected with vIRG in the absence or presence of varying concentrations of DOX or IPTG, and 2 DPI cells were imaged by fluorescence microscopy. The resulting single plaques display a wide range of EGFP expression levels, allowing both inducers to simultaneously modulate EGFP expression.

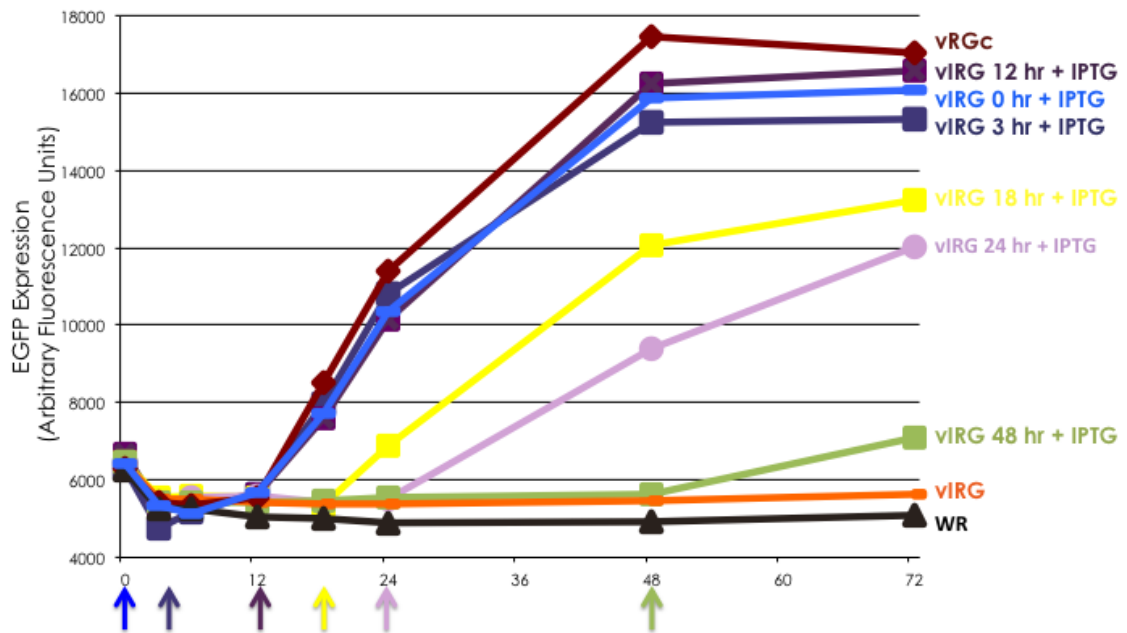


FIGURE 2.13. Kinetics of EGFP induction by vIRG. BS-C-1 cells were infected at an MOI of 1 with vIRG, WR, or vRGc and treated with 1 mM IPTG at the time points shown (+ IPTG) or left untreated. At various time points cells were imaged by fluorescence microscopy and EGFP expression was quantified. Fluorescence levels in WR- and vRGc-infected cells were not altered by addition of IPTG (data shown are only in the absence of IPTG). EGFP expression by vIRG was not affected when IPTG was added between 0 and 12 hr, but increased significantly when IPTG was added between 18 and 48 hr, demonstrating that induction can be regulated even after infection.

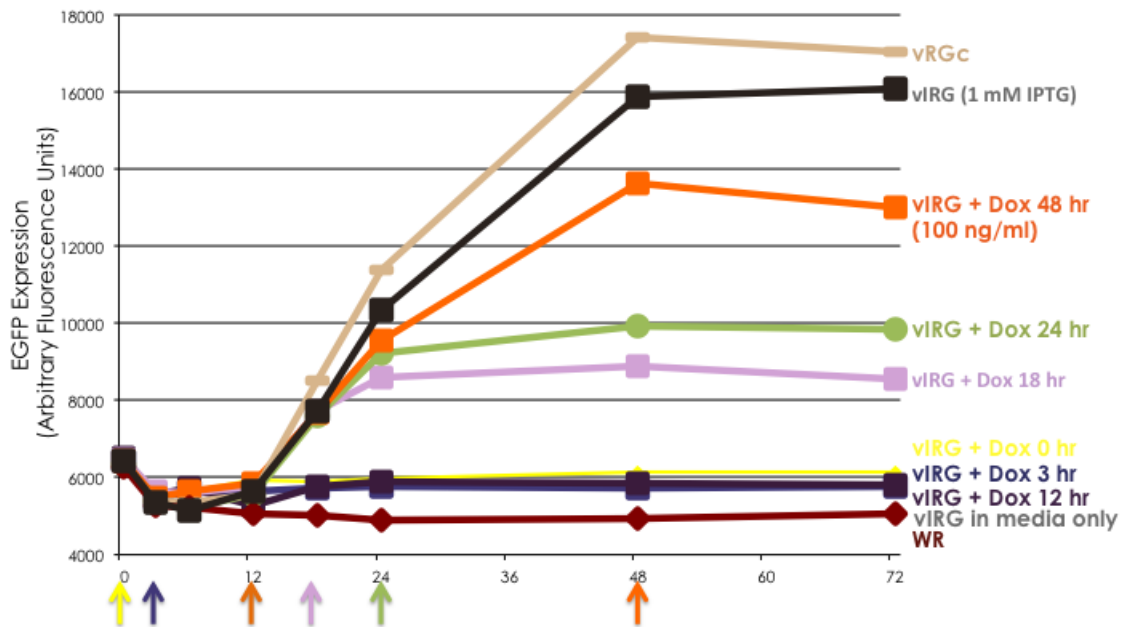


FIGURE 2.14. Kinetics of EGFP repression by vIRG. BS-C-1 cells were infected at an MOI of 1 with vIRG, WR, or vRGc in the presence of 1 mM IPTG (except when labeled medium only) and at the time points shown DOX was added to 100 ng/ml (+ DOX). At various time points cells were imaged by fluorescence microscopy and EGFP expression was quantified. Fluorescence levels in WR- and vRGc-infected cells were not altered by addition of DOX (data shown are in the presence of medium only). EGFP expression by vIRG was not affected when DOX was added between 0 and 12 hr, but decreased significantly when DOX was added between 18 and 24 hr, demonstrating that repression can be regulated even after infection.

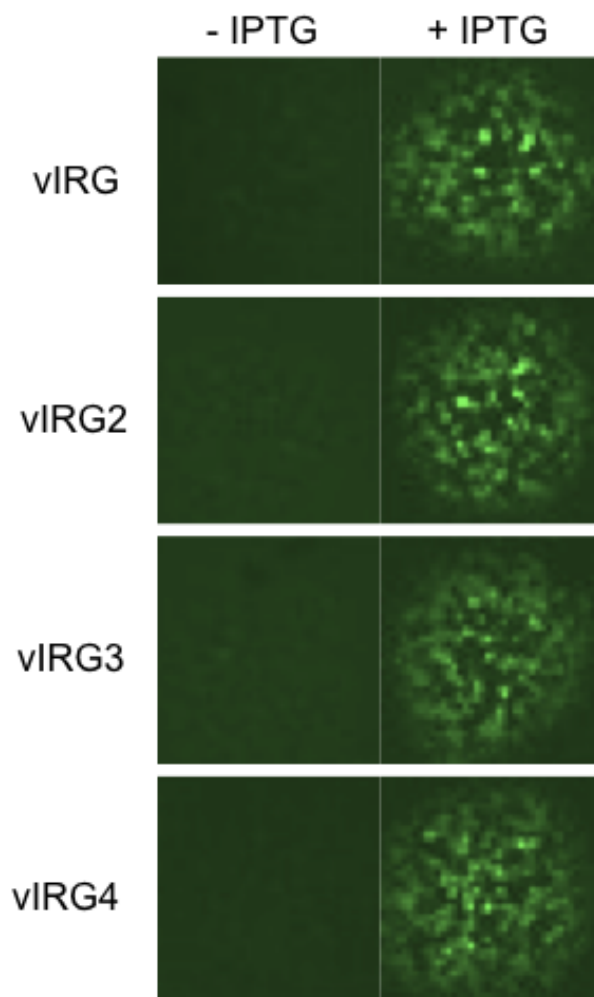


FIGURE 2.15. Addition of *tetO* operators in different numbers and arrangements does not seem to prevent leaky expression of LacI. BS-C-1 cells were infected with the different viruses in the absence (- IPTG) or presence (+ IPTG) of 5 mM IPTG, and isolated plaques were imaged by fluorescence microscopy. The levels of EGFP expression in the absence of IPTG did not differ between the different recombinant VACVs, indicating that the number and arrangement of *tetO* operators tested were not able to stop leaky expression of LacI.

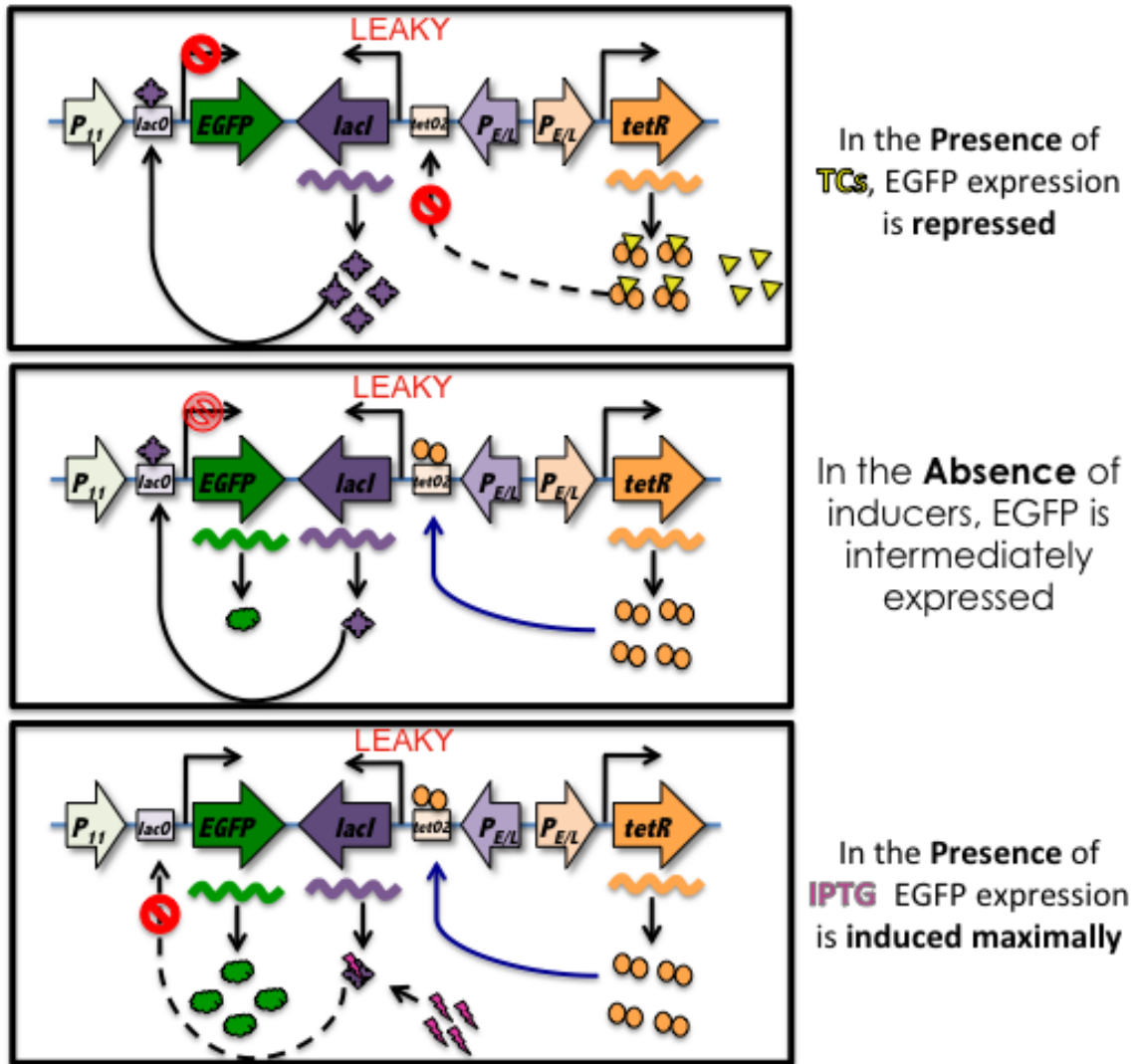


FIGURE 2.16. Schematic of the “gene circuit” mechanism underlying the control of gene expression in vIRG. The constitutive expression of *tetR* provides a continuous supply of the TetR repressor protein. In the absence of inducers (middle panel), *lacI* is expressed at low levels and interferes with the expression of EGFP. In the presence of TCs (top panel), *lacI* is expressed abundantly, repressing the expression of EGFP. In the presence of IPTG (lower panel), LacI is unable to block EGFP transcription.

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CHAPTER 3:

DEVELOPMENT OF A REPRESSIBLE VACCINIA VIRUS EXPRESSION SYSTEM ASSEMBLED WITH REVERSE *TET* REPRESSOR MUTANTS AND OTHER ELEMENTS FROM THE *TET* OPERON

3.1. ABSTRACT

A number of recombinant vaccinia viruses (VACVs) that contain elements from the tetracycline (*tet*) operon system of *E. coli* were developed to repress the expression of a reporter gene, enhanced green fluorescent protein (EGFP), in the presence of *tet* operon inducers. The recombinant VACVs constitutively expressed six mutant versions of the *tet* repressor gene (*tetR*) shown to bind *tet* operators in the presence, but not absence of inducers (reverse *tetR* genes). In the absence of tetracyclines (TCs), such as tetracycline (TET), doxycycline (DOX), and anhydrotetracycline (ATC), EGFP was expressed under the control of a VACV promoter containing a *tet* operator, albeit at different levels. In the presence of TCs, all recombinants exhibited various degrees of repression of EGFP expression, with increasing concentrations of TCs leading to EGFP repression in a dose dependent manner. All recombinants were primarily responsive to DOX, followed by ATC and TET. In the presence of DOX, EGFP expression levels were similar to a negative control virus at concentrations as low as 10 ng/ml. In light of the renewed interest for the use of VACV as vaccine and therapeutic cancer vectors, the repressible VACV expression system developed here can be used to tightly regulate genes essential for VACV replication, thus functioning as a built-in safety mechanism to conditionally control viral replication.

3.2. INTRODUCTION

Vaccinia virus (VACV) belongs to the family *Poxviridae* and has many unique characteristics that have led to its widespread use as a vaccine and therapeutic vector (Moss, 1991, Moss, 1996). In addition to its large size (200 nm × 250 nm), VACV has a 190 kb genome that encodes its own necessary transcription machinery and allows transcription to occur completely within the host cell cytoplasm. As a viral vector, VACV can carry at least 25 kb of heterologous DNA (Smith and Moss, 1983), and it can elicit strong humoral and cell-mediated immune responses (Mackett et al., 1982). VACV has become most notable for its use as the smallpox vaccine that eradicated smallpox worldwide (Fenner et al., 1988, World Health Organization. Global Commission for the Certification of Smallpox Eradication and World Health Organization, 1980). After this great achievement, VACV became one of the most characterized viruses, allowing the development of heterologous vaccines and cancer therapeutics.

The use of VACV as a replication competent virus is associated with severe adverse events, and there is a growing segment of the population for which its use is contraindicated (Cono et al., 2003, Lane and Goldstein, 2003). Those that are immunosuppressed, have (or had) atopic dermatitis and other skin conditions, and those with heart conditions fall within the contraindicated population. This is a significant limitation and is problematic for various reasons. Amidst the fears of bioterrorism and the re-emergence of variola virus or related pathogenic poxviruses (Dhawan et al., 2001, Di Giulio and Eckburg, 2004, Nalca et al., 2005, Gottschalk and Preiser, 2005), the US has established a stockpile of the smallpox vaccine (Monath et al., 2004, Greenberg et al., 2005, Greenberg and Kennedy, 2008). The currently stockpiled vaccine (ACAM2000),

passaged in cell culture, has improved sterility (Greenberg and Kennedy, 2008, Monath et al., 2004) over the previously used smallpox vaccine (Dryvax), produced in the skin of calves (Rotz et al., 2001). However, the adverse events associated with virus replication are still a significant concern with ACAM2000. One way to address these safety concerns is to develop replication incompetent VACVs, such as MVA (Modified Vaccinia Ankara). MVA-BN or IMVAMUNE is a potential next-generation smallpox vaccine that is currently on fast-track clinical trials (Kennedy and Greenberg, 2009). However, MVA elicits lower immune responses after a single immunization (thus requiring multiple doses to elicit immune responses comparable to Dryvax or ACAM2000) and it is replication-defective, thus unable to produce the “take”, the only known correlate of protection against smallpox.

Thus, there is a need for alternative approaches to increase the safety of VACV while maintaining its immunogenicity and its ability to produce a take. One approach that meets these criteria is to place a gene essential to viral replication under the control of a repressible system, which could serve as a built-in safety system. In cases where adverse events were to occur, transcription of a gene essential for viral replication could be blocked upon administration of an inducer, thus stopping the replication of the virus and most importantly, the progression of the adverse event.

In this study, elements from the *tet* operon of *E. coli* and reverse mutants of the *tetR* (*revtetR*) gene were used to generate recombinant VACVs that repress the expression of a reporter gene, enhanced green fluorescent protein (EGFP), in the presence of tetracyclines (TCs).

3.3. MATERIALS AND METHODS

Viruses and Cell Culture. African green monkey kidney (BS-C-1) and human (HeLa S3) cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% TET tested fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) at 37°C in 5% CO₂. A clone derived from the Western Reserve (WR) strain of VACV (ATCC VR-2035) and all recombinant VACVs were propagated in BS-C-1 and HeLa S3 cells and titered by plaque assay in BS-C-1 cells.

Transfer Vectors. The construction of the vRG0 transfer vector is detailed in Figure 3.1. Briefly, the *NheI* and *XmaI* fragment of pAT008, a designed synthetic plasmid (DNA2.0, Menlo Park, CA) that contains the *revtetR* (L17G) gene (Scholz et al., 2004), was subcloned into the same sites of pSMART11 (Weber et al., 2007). The resulting plasmid (pAT013) contains the *revtetR* gene under the control of the synthetic early/late VACV P_{E/L} promoter, the DsRed gene under the control of the natural late VACV P₁₁ promoter (back-to-back with the P_{E/L}) with a 19 bp *tetO*₂ placed directly downstream from P₁₁ and upstream from the transcriptional start site, and the *gpt-gus* fusion gene under the control of the synthetic VACV P_{SeI} promoter (Hammond et al., 1997), flanked by segments of the VACV thymidine kinase (TK) gene (left, TK_L and right, TK_R), which allows for homologous recombination with the VACV TK genomic region. The final transfer vector pAT020 was generated by transferring the EGFP gene from pAT010 into the *BglII* and *BspEI* sites of pAT013. A control transfer vector (pAT018) for the generation of a recombinant VACV constitutively expressing EGFP under the P₁₁ promoter (vRGc) was

generated by transferring the *revtetR* gene into plasmid pSMART10 (Weber et al., 2007), and by replacing the DsRed gene with the EGFP gene as described above. Additionally, transfer vectors with different mutants of the *revtetR* gene (Table 3.1) were generated by subcloning the mutant *revtetR* synthetic genes (DNA2.0) into the *NheI* and *XmaI* sites in pAT020.

Generation of Recombinant VACVs. The recombinant VACVs vRG0, vRG1, vRG2, vRG3, vRG4, vRG5 (Table 3.1), and vRGc (a positive control virus without *tetO₂*, thus constitutively expressing EGFP under the P₁₁ promoter) were generated via standard homologous recombination by transfection of the transfer vectors into BS-C-1 cell monolayers infected with VACV strain WR (also used as the negative control) at a multiplicity of infection (MOI) of 0.05 plaque-forming units (PFU)/cell. Recombinant VACVs were plaque purified in selection medium (25 µg/ml mycophenolic acid, 250 µg/ml xanthine, 15 µg/ml hypoxanthine); plaques were visualized either with substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) or by selecting and collecting only EGFP⁺ expressing plaques detected via fluorescence microscopy. The purity of the recombinant VACVs (absence of parental virus) was confirmed by checking multiple dilutions of stocks in selection-free media for the presence of non-fluorescent (EGFP⁻) plaques. High titer stocks of each recombinant VACV were generated by infecting HeLa S3 cells at an MOI of 0.1. Infected cells were harvested 3 days post-infection (DPI) by centrifugation at 200 × g for 10 min. Cells were then lysed by freezing and thawing, sonicated, and trypsinized. Finally, cell lysates were clarified to remove contaminating cell debris by a second round of sonication and centrifugation at 200 × g for 5 min.

Determination of EGFP Expression. BS-C-1 cell monolayers in multiwell culture plates were infected with the VACVs (30 PFU or an MOI of 1) in the presence of medium only or various concentrations of anhydrotetracycline (ATC; with or without various concentrations of MgCl_2 , allowed to form a complex for 1 hr before use), tetracycline (TET), or doxycycline (DOX). At 2 DPI, EGFP expression by isolated plaques or infected cells were determined by quantitative fluorescence microscopy (as described below). In certain instances, cell monolayers were washed with $1\times$ phosphate-buffered saline (PBS) pH 7.2, resuspended in equal parts of $1\times$ PBS and 2% neutral buffered formalin (1% final concentration), and analyzed with a fluorescence plate reader (as described below) within 24 hr.

Imaging and Image Quantification. Infected cells and plaques were imaged using an inverted fluorescence microscope (Axio Observer D1, Carl Zeiss, Thornwood, NY) with or without a green bandpass filter (XF100-2, Omega Optical, Brattleboro, VT). EGFP expression levels were quantified with the AxioVision software, release 4.8.1 (Carl Zeiss).

Fluorescence Quantification with a Microplate Reader. The fluorescence readings of infected cells were obtained with a fluorescence plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek Instruments, Winooski, VT) using a 485/20 nm excitation and a 528/20 nm emission filter pair. EGFP expression levels are quantified with the KC4 v3.4 software (BioTek Instruments).

3.4. RESULTS

vRG0 Expresses EGFP at High Levels in the Absence of TCs. The first mutant *revtetR* tested in this system was based on a *tetR* mutant with a single amino acid change at position 17 that changes leucine to glycine (L17G) (Scholz et al., 2004). Although this L17G revTetR mutant was characterized in bacteria (*E. coli*), it was appealing to test initially in this system, since a single mutation is less likely to alter other important regions on the repressor protein. In addition, this mutant was characterized as a single domain protein, unlike most other *revtetR* mutants that were characterized as fusion proteins with the VP16 transcriptional activator from herpes simplex virus. Moreover, it is important to note that ATC (a TC-derivative) was used to characterize the L17G mutant in *E. coli*.

In the absence of TCs, BS-C-1 cells infected with vRG0, a positive control virus (vRGc) or a negative control virus (WR) displayed the expected cytopathic effect (CPE) 2 DPI when observed under brightfield microscopy. Additionally, vRG0 and vRGc plaques displayed similar high levels of EGFP expression under fluorescence microscopy, while WR-infected cells did not (Figure 3.2).

EGFP Expression Levels by vRG0 were not Repressed even in the Presence of High Concentrations of TCs. To test whether vRG0 was repressible by TCs, BS-C-1 cells were infected with vRG0 in the presence of increasing concentrations of ATC, TET, or DOX. The CPE observed by brightfield microscopy 2 DPI was indistinguishable from the CPE observed from cells treated with ATC and 10 µg/ml or less of TET or DOX

(Figure 3.3). However, cells infected in the presence of 100 $\mu\text{g/ml}$ of TET or DOX exhibited morphology associated with cell toxicity, and as a result the plaques were smaller in comparison to infected cells treated with lower concentrations of TET or DOX. Cells infected in the presence of ATC did not incur any toxicity because lower concentrations were used in comparison to the TET and DOX, as ATC has the highest affinity for revTetR (Scholz et al., 2004). Increasing concentrations of TCs did not decrease EGFP expression in vRG0 plaques when observed by fluorescence microscopy. In the presence of 100 $\mu\text{g/ml}$ of TET or DOX, infected cells exhibited lower EGFP expression, probably due to cell toxicity.

Since TCs complexed to MgCl_2 bind to the TetR repressor with higher affinity than TCs alone (a commonly used strategy when studying induced conformation of the repressor by x-ray crystallography), we decided to incubate ATC with MgCl_2 to allow for complex formation (Kamionka et al., 2004, Scholz et al., 2003). Cells infected with vRG0 in the presence of varying concentrations of ATC- MgCl_2 displayed typical CPE 2 DPI at 20 mM or below of MgCl_2 , while at the highest concentration of MgCl_2 (100 mM MgCl_2), the cells exhibited toxicity-associated morphology (data not shown). When plaques were observed by fluorescence microscopy, there was no EGFP repression in the presence of increasing concentrations of the ATC- MgCl_2 complex (Figure 3.4). In addition, at the highest concentrations of ATC- MgCl_2 complex tested (100 mM), no fluorescent plaques were observed, probably as a result of MgCl_2 cell toxicity. Thus, we concluded that that vRG0 was not repressible and decided to test new *revtetR* mutants (Table 3.1).

RevTetR mutants Lead to Different EGFP Expression Levels in the Absence of TCs.

Five new recombinant VACVs (vRG1, vRG2, vRG3, vRG4, and vRG5) were generated (Table 3.1), each expressing a different mutant *tetR* gene previously shown to display the reverse phenotype in eukaryotic systems when fused to a VP16 domain from herpes simplex virus that induces gene expression. Since the transactivator will not function in VACV, as VACV relies on its own specialized transcription machinery, we tested the revTetR mutants without the VP16 transactivator. Additionally, these mutants were initially characterized with the TC derivative, DOX; TET and DOX between 0.1 to 1 µg/ml was successfully used in TC-inducible VACV expression systems (Trakman et al., 2000, Weber et al. 2007). Furthermore, DOX is an FDA approved, broad-spectrum drug that exhibits antimicrobial and off target activities (Greenwald et al., 2001)

In the absence of TCs, BS-C-1 cells infected with the VACVs displayed the expected CPE 2 DPI under brightfield microscopy. When observed under fluorescence microscopy, vRG5, vRG0, and vRGc plaques displayed similar high levels of EGFP expression (Figure 3.5). The other recombinants had varying lower levels of expression, with vRG3 and vRG4 having equal levels of EGFP expression, vRG2 less, and vRG1 least.

RevTetR mutants Lead to Different Repression of EGFP Expression Levels in the Presence of TCs. BS-C-1 cells were infected with the different VACVs at an MOI of 1 in the absence or presence of varying concentrations of TCs. Two DPI cells were imaged by brightfield and fluorescence microscopy and EGFP expression was subsequently quantified. Based on fluorescence microscopy images (Figure 3.6) and quantification

(Figures 3.7, 3.8, and 3.9), all revTetR mutants displayed varying levels of EGFP repression, generally being most sensitive to DOX, followed by ATC and then TET. vRG0 was repressible, but EGFP expression remained high even at the highest concentrations of TCs tested. In the presence of DOX, only vRG1 and vRG2 fully repressed EGFP expression to the levels of the negative control WR at 0.1 $\mu\text{g/ml}$, although EGFP expression levels by vRG1 in the absence of TCs were the lowest (Figure 3.9). In addition, vRG4 was able to be fully repressed at 1 $\mu\text{g/ml}$ of DOX, while vRG3 was fully repressed at 10 $\mu\text{g/ml}$ of DOX. In the absence of TCs, vRG5 was able to express EGFP at the same level as vRGc, although it was not able to become fully repressed based on fluorescence imaging quantification.

Fluorescence Multiwell Reader Displays Higher Sensitivity at Low Levels of EGFP

Expression. To potentially increase the sensitivity and reproducibility of the fluorescence quantification and to better ascertain the repressible systems that exhibit full repression, a fluorescence plate reader (fluorometer) was employed. BS-C-1 cells were infected with the VACVs at an MOI of 1 in multiwell plates in the absence or presence of increasing concentrations of TCs, and 2 DPI cells were fixed and read on the multiwell fluorescence reader (Figure 3.10, 3.11, and 3.12). The results follow essentially the same trends observed by fluorescence imaging. In addition, it seems that lower levels of EGFP expression were detected with increased sensitivity, although there was more variance at higher levels of EGFP expression. Thus, for the purposes of determining EGFP repression by the recombinant VACVs, the fluorometer seems more appropriate as it is more sensitive at lower levels of expression.

Full Repression of EGFP Expression is Achievable in VACV. To determine the TC concentration that fully represses EGFP expression, BS-C-1 cells were infected with the VACVs in the absence or presence of increasing concentrations of TCs, and 2 DPI cells were fixed and read on the fluorescence multiwell reader. In the presence of increasing concentrations of ATC (0.1 ng/ml – 1,000 ng/ml), vRG1-vRG5 showed a repressible dose response (Figure 3.13). vRG1 was fully repressed to the levels of the negative control WR in the presence of 100 ng/ml ATC, while vRG2, vRG3, and vRG4 were fully repressed in the presence of 1,000 ng/ml ATC. Only vRG5 and vRG0 were not fully repressed at 1,000 ng/ml of ATC.

In the presence of increasing concentrations of TET (1 ng/ml – 10,000 ng/ml), the expression in vRG1-vRG5 also showed a repressible dose response (Fig. 3.14). However, vRG1 was the only recombinant that became fully repressed to the levels of WR. In the presence of increasing concentrations of DOX (1 ng/ml – 10,000 ng/ml), all recombinant VACVs show a repressible dose response (Figure 3.15). With the exception of vRG0 and vRG5, all other recombinants were fully repressed by concentrations of DOX within the range tested; however, some recombinant VACVs are more sensitive to DOX than others. vRG1 was fully repressed at 10 ng/ml of DOX, vRG2 was next at 100 ng/ml, and vRG3 and vRG4 were next at 1,000 ng/ml. At the highest DOX concentration tested (10,000 ng/ml), vRG5 seems to be mostly, but not completely repressed.

3.5. DISCUSSION AND CONCLUSIONS

All of the revTetR repressor mutants tested in this VACV expression system displayed a repressible phenotype in the presence of TCs, albeit with unique phenotypes. For example, vRG0 (with the single L17G *tetR* mutant) was only marginally repressible, while the recombinant VACV most sensitive to TCs (vRG1) was also the one with the lowest level of EGFP expression in the absence of TCs. As a general trend, the higher the level of EGFP expression in the absence of TCs, the higher the TC concentration needed to completely repress the system.

An aspect that can be tested with this repressible system is whether EGFP expression can be repressed even after infection. As it was demonstrated in Chapter 2 in a modulatory expression system that is both repressible and inducible, EGFP expression could be stopped in the presence of DOX at various time points post-infection (Titong and Verardi, unpublished data). Similar experiments could demonstrate if this is true in this revTetR based repression system.

Another interesting aspect of this work is the sensitivity of fluorescence quantification. In this repressible system, the main goal is to establish levels of repression, in particular how close EGFP expression levels are to negative control levels. The use of quantitative fluorescence imaging was reliable for the measurement of high fluorescence levels, but the multiwell fluorometer was considerably more sensitive for measurements of low levels of fluorescence, despite having greater variation in the higher ranges of expression.

There are many applications for which the different revTetR versions in these repressible systems can be used. In some cases, genes may not necessarily have to be

expressed at high levels to achieve the necessary phenotype. In general, enzymes are an example of a class of genes do not need high levels of expression, in which case the vRG1 or vRG2 systems could be appropriate to use. In other cases, such as for the expression of virion structural proteins, higher levels of expression may be needed and vRG3, vRG4, or vRG5 may be more applicable as each exhibits increasing levels of expression in the absence of TCs.

Increasing the safety profile of VACV vectors can be used for the development of the next-generation smallpox vaccines, human and animal VACV-vectored vaccines, as well as immune and oncolytic therapies. More specifically, a new generation of smallpox vaccine can be generated by placing a gene essential for viral replication under the control of a repressible system. This next-generation smallpox vaccine would not be inferior to previous vaccines as it would replicate in the absence of TCs and it would be functionally identical to Dryvax or ACAM2000 and produce the only established correlate of protection (the take). Importantly, this new vaccine would have an enhanced safety profile as this built-in safety mechanism could stop viral replication after administration of FDA approved TCs (such as DOX) in the event of adverse reactions to vaccination.

Another example in which a repressible system would be extremely useful is the current Raboral V-RG (Merial, Duluth, GA) sylvatic rabies vaccine, a VACV-vectored vaccine expressing the glycoprotein of rabies that is widely used to control sylvatic rabies (Pastoret and Brochier, 1996, Brochier et al., 1996). The means in which this vaccine is delivered poses great risks, especially to those with contraindications against vaccination with VACV. Vaccine baits are dropped in wooded areas, in some cases these areas are

very near urbanized settings (Reynolds et al., 2007). Thus, humans have the potential to be exposed to the live vaccine (Centers for Disease Control and Prevention (CDC), 2009). By using our built-in safety repressible mechanism, a newer generation of recombinant rabies vaccines could be developed. Should a contraindicated individual accidentally come into contact with vaccine baits, any adverse events due to VACV infection could be stopped with the administration of TCs.

VACV on its own has an array of practical applications and has led to great achievements in medicine. We have successfully described an unique repressible system that can be built into VACV and used to greatly improve its safety profile as a viral vector for vaccines and immunotherapeutics.

3.6. FIGURES AND TABLE

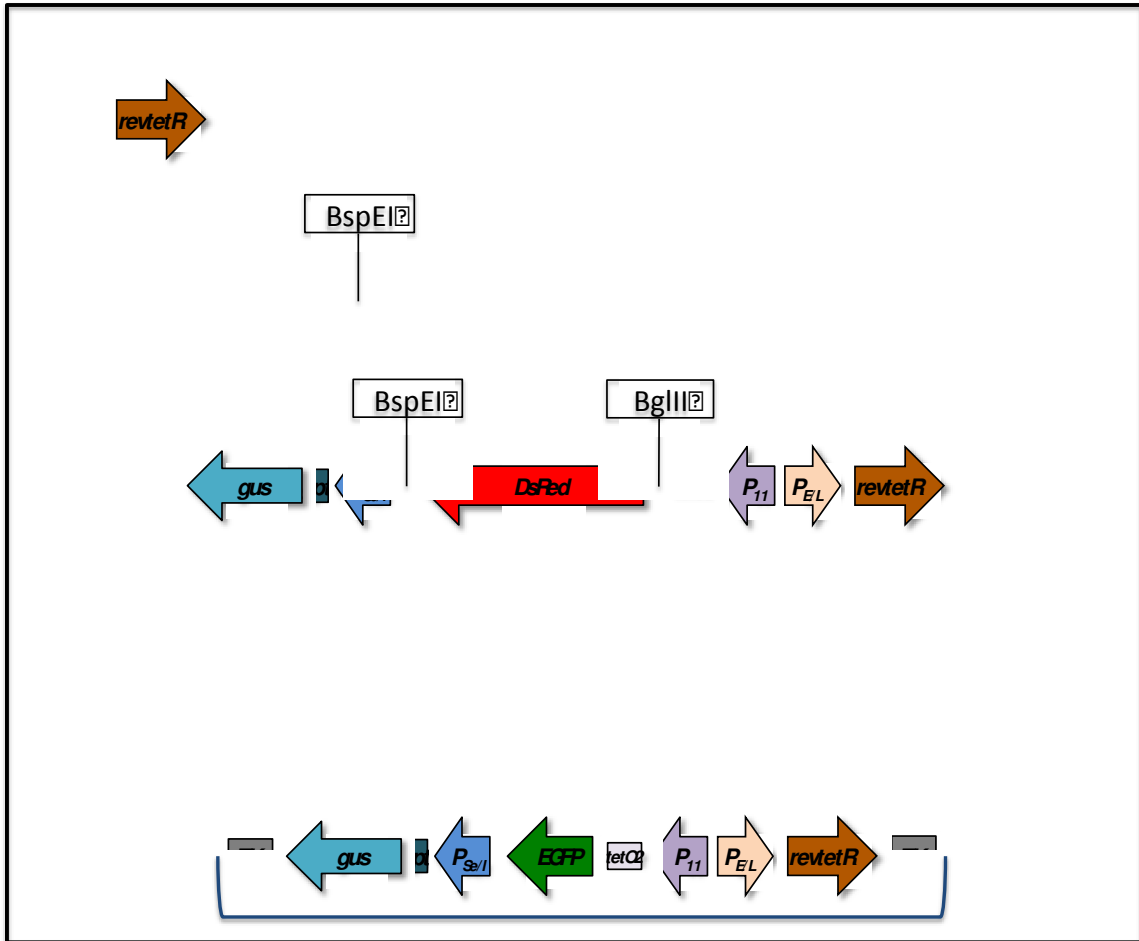


FIGURE 3.1. Construction of the transfer vector pAT015 for the generation of vRG0. The cloning steps leading to the generation of the final transfer vector are shown. pAT020 contains the *gpt-gus* fusion gene under the control of a synthetic early/late VACV promoter (P_{Sel}), the EGFP gene under the control of an engineered late P_{11} promoter with a *tetO₂* sequence located directly downstream, the *revtetR* gene under the control of a constitutive strong synthetic early/late promoter (P_{EL}), all flanked by TK_L and TK_R sequences that direct recombination with the TK gene of VACV.

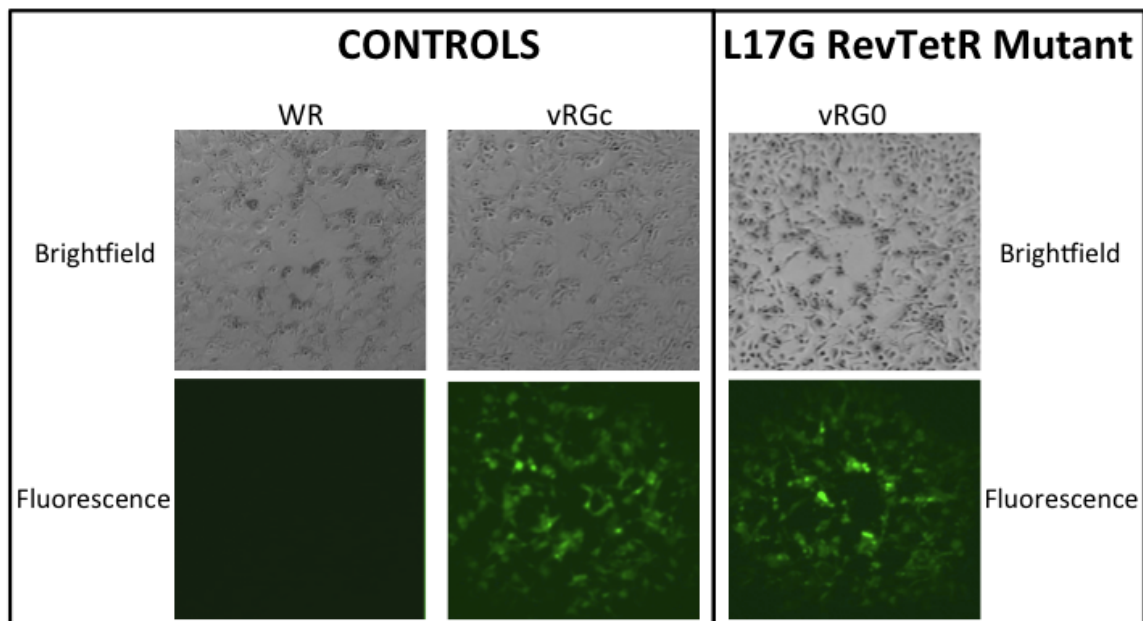
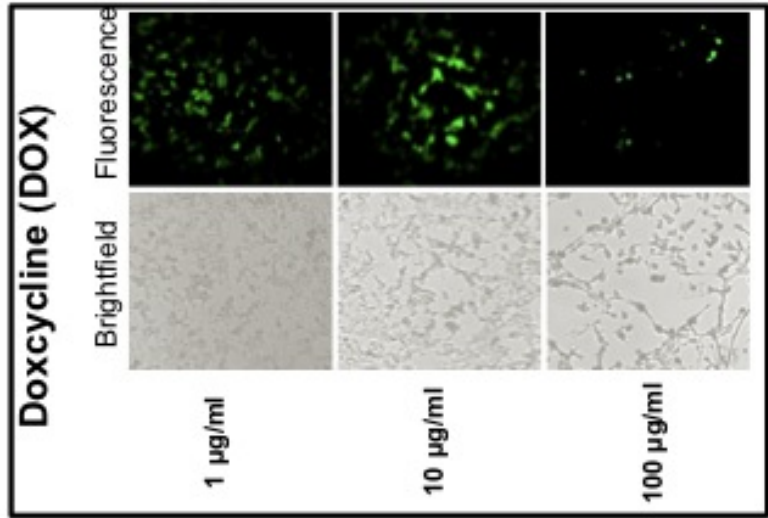
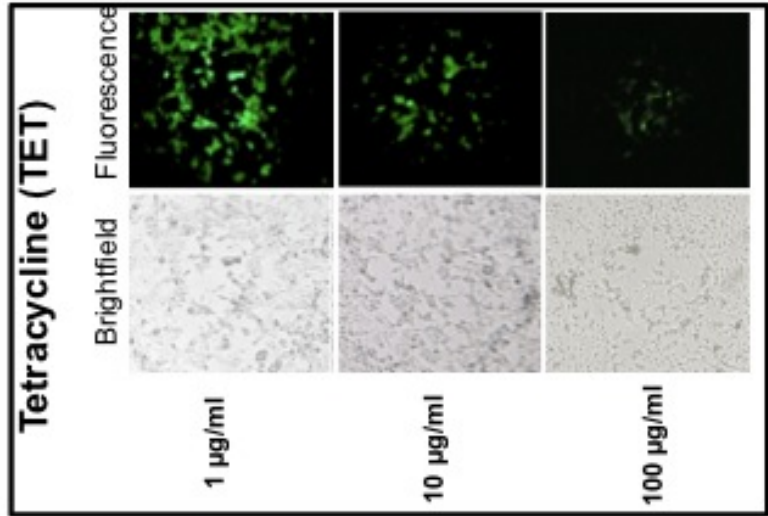
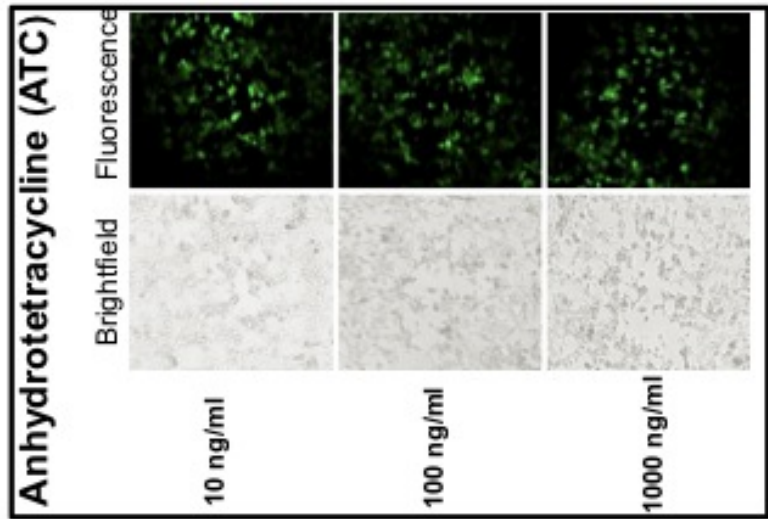
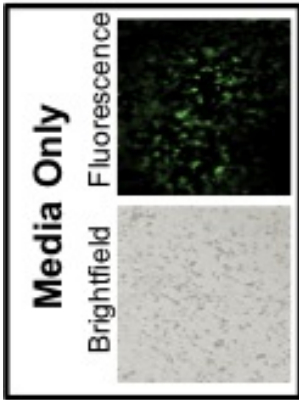


FIGURE 3.2. vRG0 expresses EGFP at high levels in the absence of TCs. BS-C-1 cells infected with WR, vRGc, or vRG0 in the absence of TCs displayed typical CPE 2 DPI, and single isolated plaques were imaged by brightfield and fluorescence microscopy. Plaques from vRG0 and vRGc (positive control virus) displayed similar high levels of EGFP expression, while WR (negative control virus) did not.

FIGURE 3.3. EGFP expression levels by vRG0 were not repressed in the presence of TCs. BS-C-1 cells infected with vRG0 in the presence of TCs displayed typical CPE 2 DPI in all concentrations of ATC or 10 µg/ml or less of TET or DOX. However, cells infected with 100 µg/ml of TET or DOX exhibited morphology associated with cell toxicity and as a result the plaques were smaller. Increasing concentrations of TCs did not decrease EGFP expression in vRG0 plaques when observed by fluorescence microscopy.



[TCS]

FIGURE 3.4. EGFP expression is not repressed in cells infected with vRG0 in the presence of varying concentrations of the ATC-MgCl₂ complex. BS-C-1 cells infected with vRG0 in the presence of ATC-MgCl₂ displayed typical CPE 2 DPI in concentrations of 20 mM MgCl₂ or less. However, cells infected with 100 mM of MgCl₂ exhibited morphology associated with toxicity and as a result a normal infection did not occur in comparison to lower concentrations (brightfield images not shown). Regardless, EGFP expression by vRG0 was not repressed by ATC-MgCl₂.

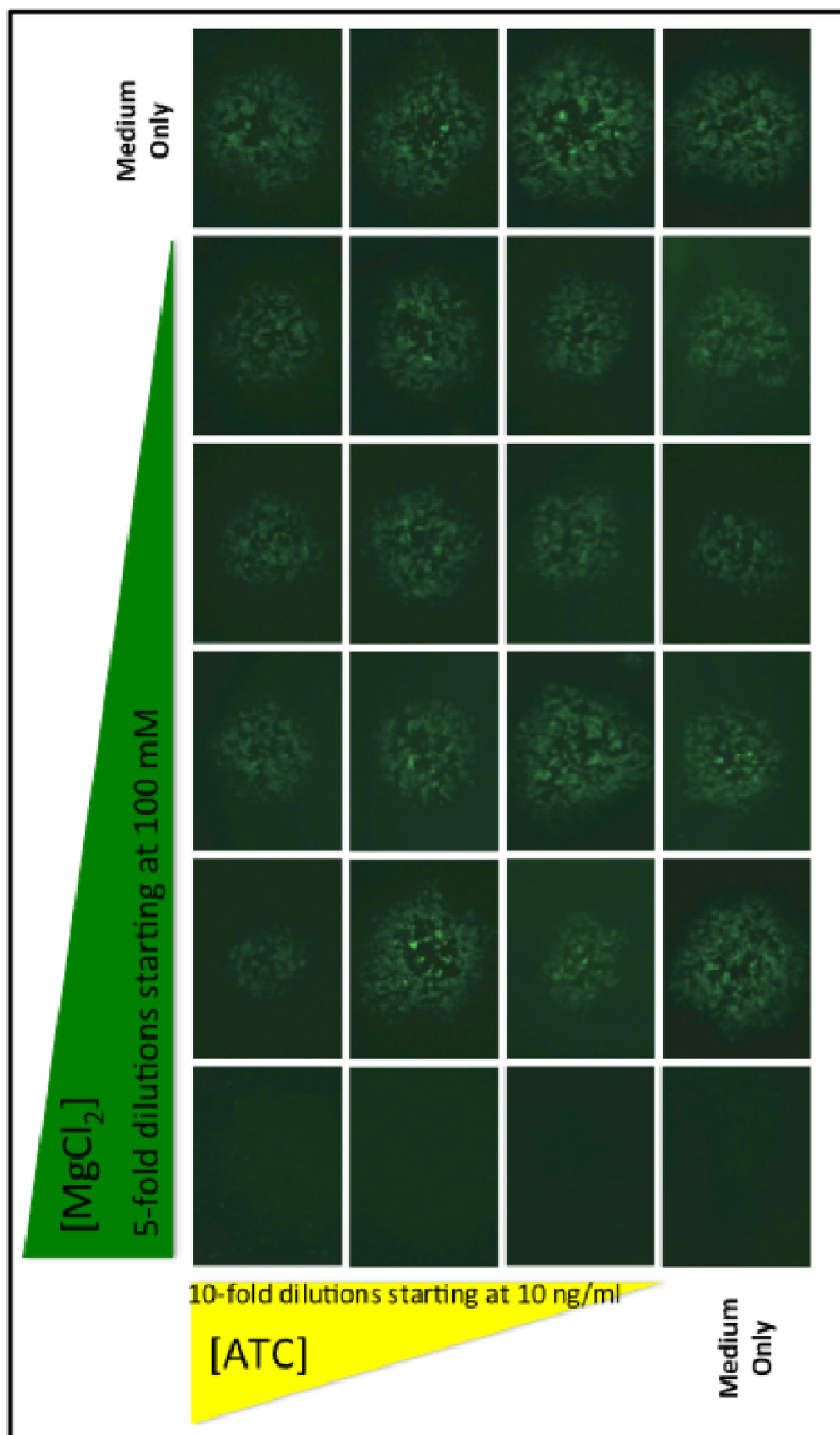


FIGURE 3.5. RevTetR mutants lead to different EGFP expression levels in the absence of TCs. BS-C-1 cells infected with the VACVs in the absence of TCs displayed typical CPE 2 DPI, and single isolated plaques were imaged by brightfield and fluorescence microscopy. vRG5, vRG0 and vRGc plaques displayed similar high fluorescence levels. The other recombinants exhibited decreasing levels of EGFP expression in the following order, vRG3 and vRG4 were equal followed by vRG2 and then by vRG1.

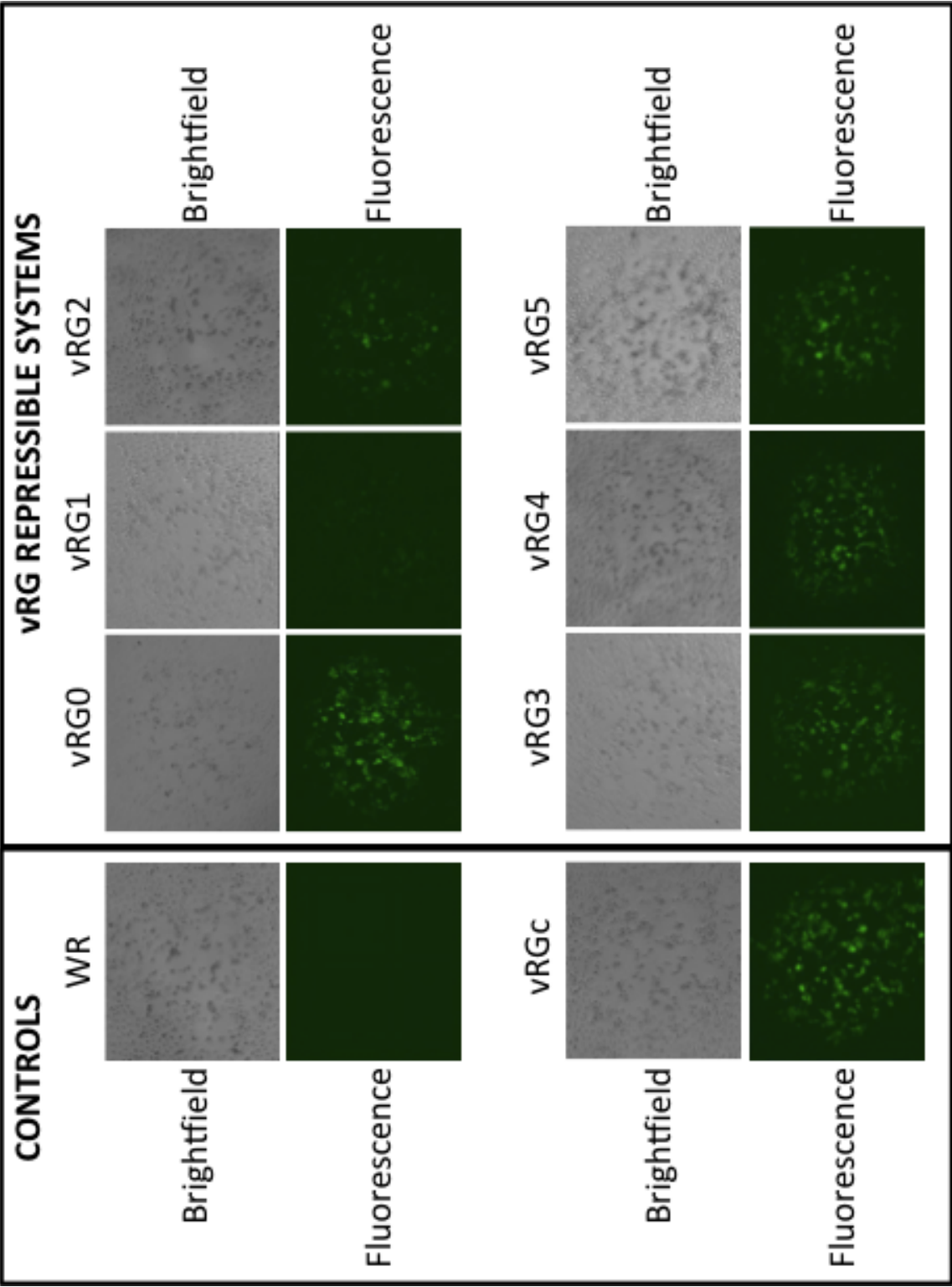


FIGURE 3.6. EGFP expression is repressed in the presence of increasing concentrations of TCs. BS-C-1 cells were infected at an MOI of 1 with the VACVs in the absence or presence of TCs and imaged by fluorescence microscopy 2 DPI. With the exception of vRG0, EGFP expression was repressed in all other recombinant VACVs, although not all expressed EGFP at high levels in the presence of TCs.

FIGURE 3.7. EGFP expression is repressed in the presence of increasing concentrations of ATC when measured by fluorescence imaging quantification.

Fluorescence levels of cells previously imaged (Figure 3.6) were quantified. Cells infected with vRG1 to vRG5 show repression of EGFP expression with increasing concentrations of ATC. vRG0 displayed minimal repression even at the highest concentration of ATC tested. Error bars are SEM, n=3.

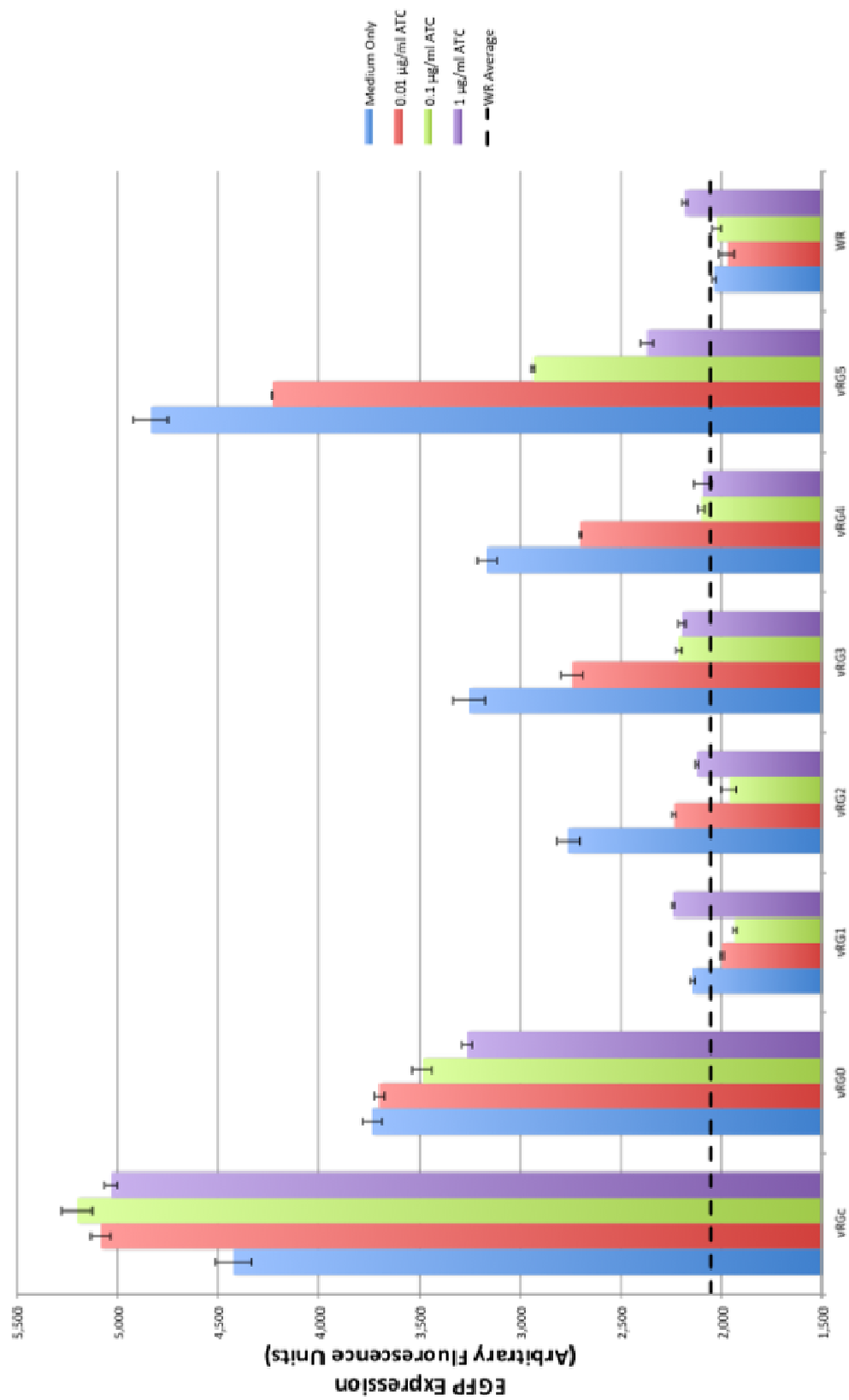


FIGURE 3.8. EGFP expression is repressed in the presence of increasing concentrations of TET when measured by fluorescence imaging quantification.

Fluorescence levels of cells previously imaged (Figure 3.6) were quantified. Cells infected with vRG1 to vRG5 show repression of EGFP expression with increasing concentrations of TET. vRG0 displayed minimal repression even at the highest concentration of TET tested. Error bars are SEM, n=3.

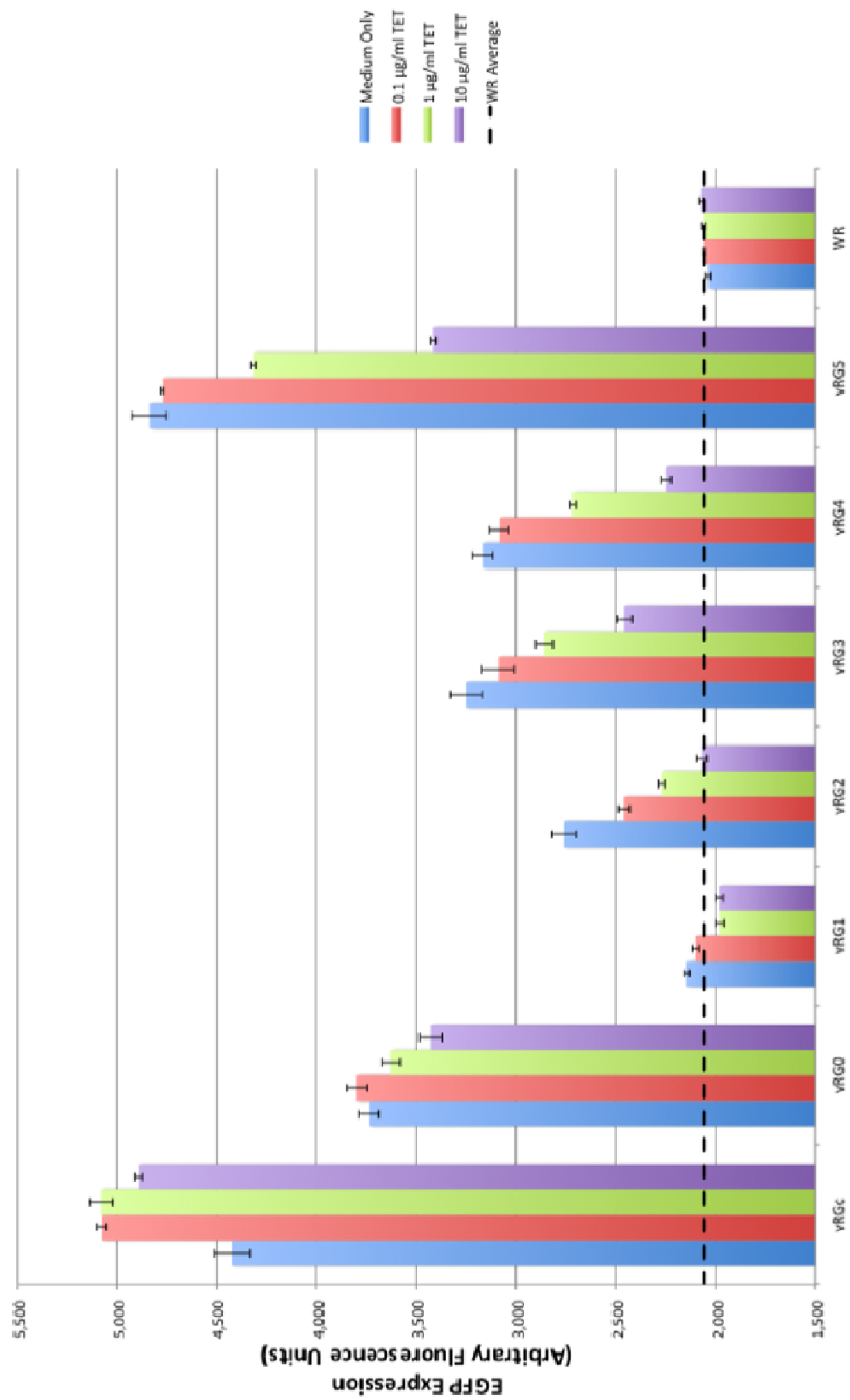


FIGURE 3.9. EGFP expression is repressed in the presence of increasing concentrations of DOX when measured by fluorescence imaging quantification.

Fluorescence levels of cells previously imaged (Figure 3.6) were quantified. Cells infected with vRG1 to vRG5 show repression of EGFP expression with increasing concentrations of DOX. Expression by vRG1, vRG2, vRG3, and vRG4 is repressible to the levels of the negative control WR. vRG0 displayed minimal repression even at the highest concentration of DOX tested. Error bars are SEM, n=3.

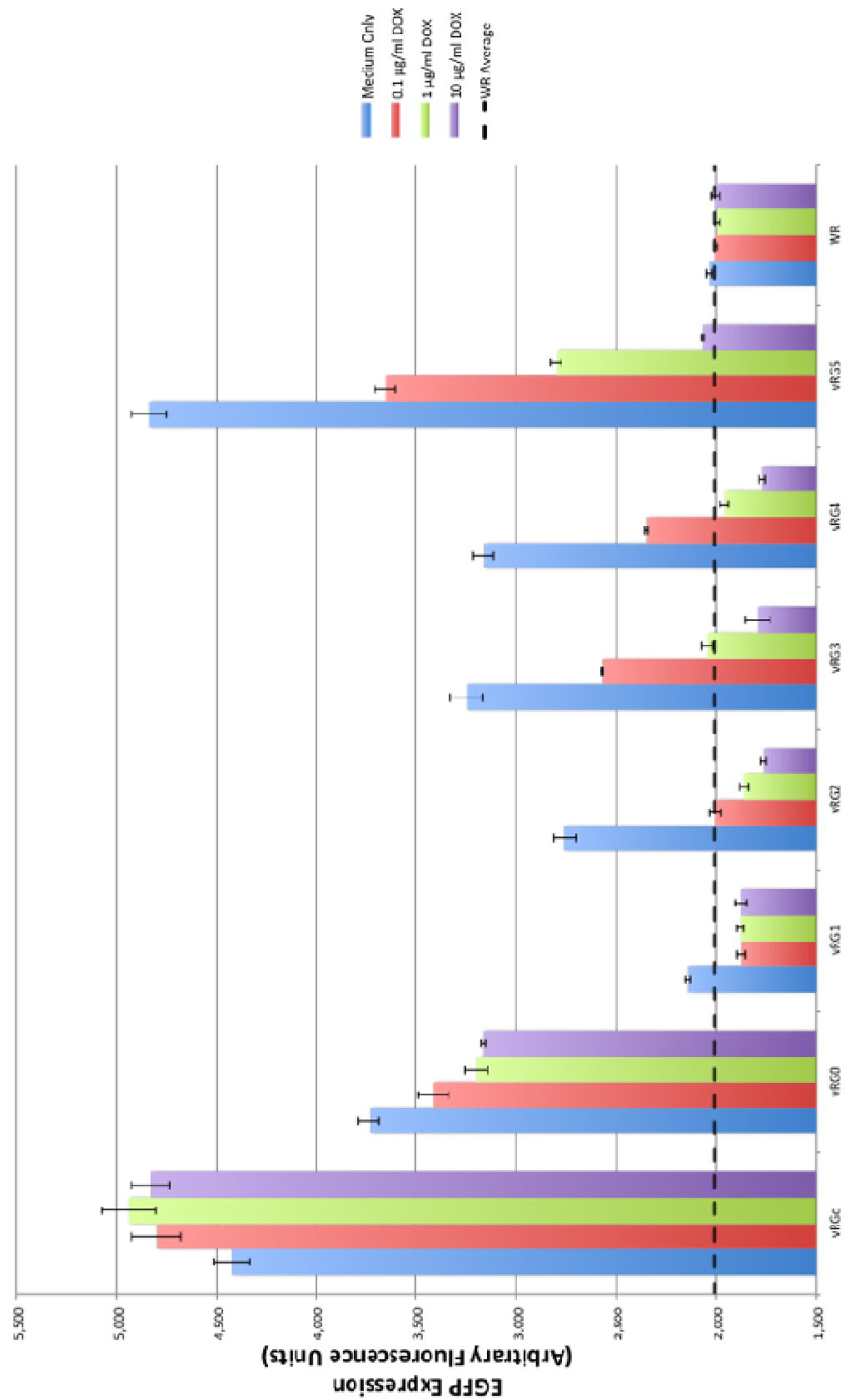


FIGURE 3.10. EGFP expression is repressed in the presence of increasing concentrations of ATC when measured by a multiwell fluorescence reader. In this preliminary study, BS-C-1 cells were infected at an MOI of 1 with the VACVs in the absence or presence of ATC and 2 DPI cells were fixed and quantified with a fluorometer. EGFP expression levels were repressed in the presence of increasing concentrations of ATC. Error bars are SEM, n=2.

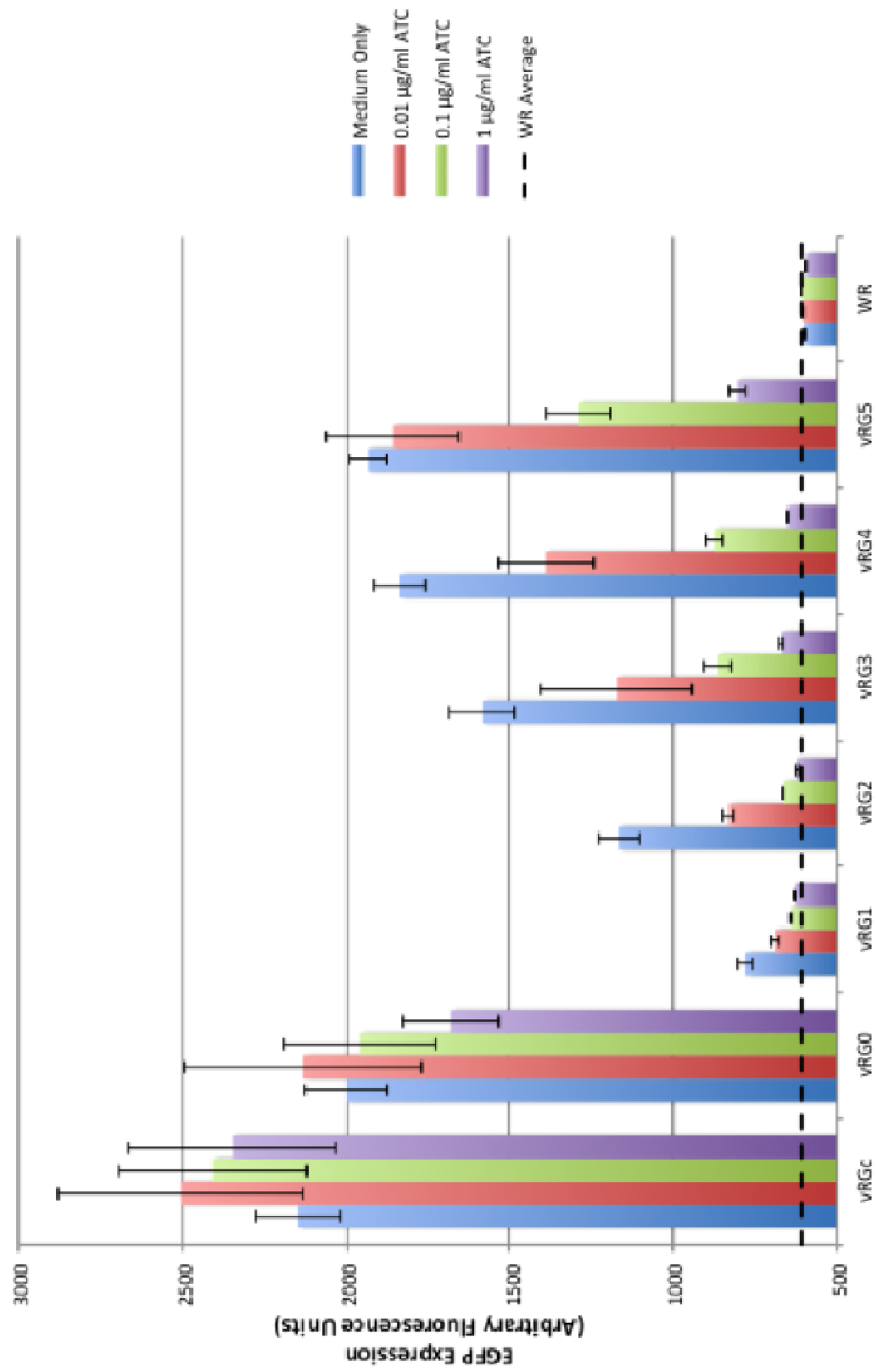


FIGURE 3.11. EGFP expression is repressed in the presence of increasing concentrations of TET when measured by a multiwell fluorescence reader. In this preliminary study, BS-C-1 cells were infected at an MOI of 1 with the VACVs in the absence or presence of TET and 2 DPI cells were fixed and quantified with a fluorometer. EGFP expression levels were repressed in the presence of increasing concentrations of TET. Error bars are SEM, n=2.

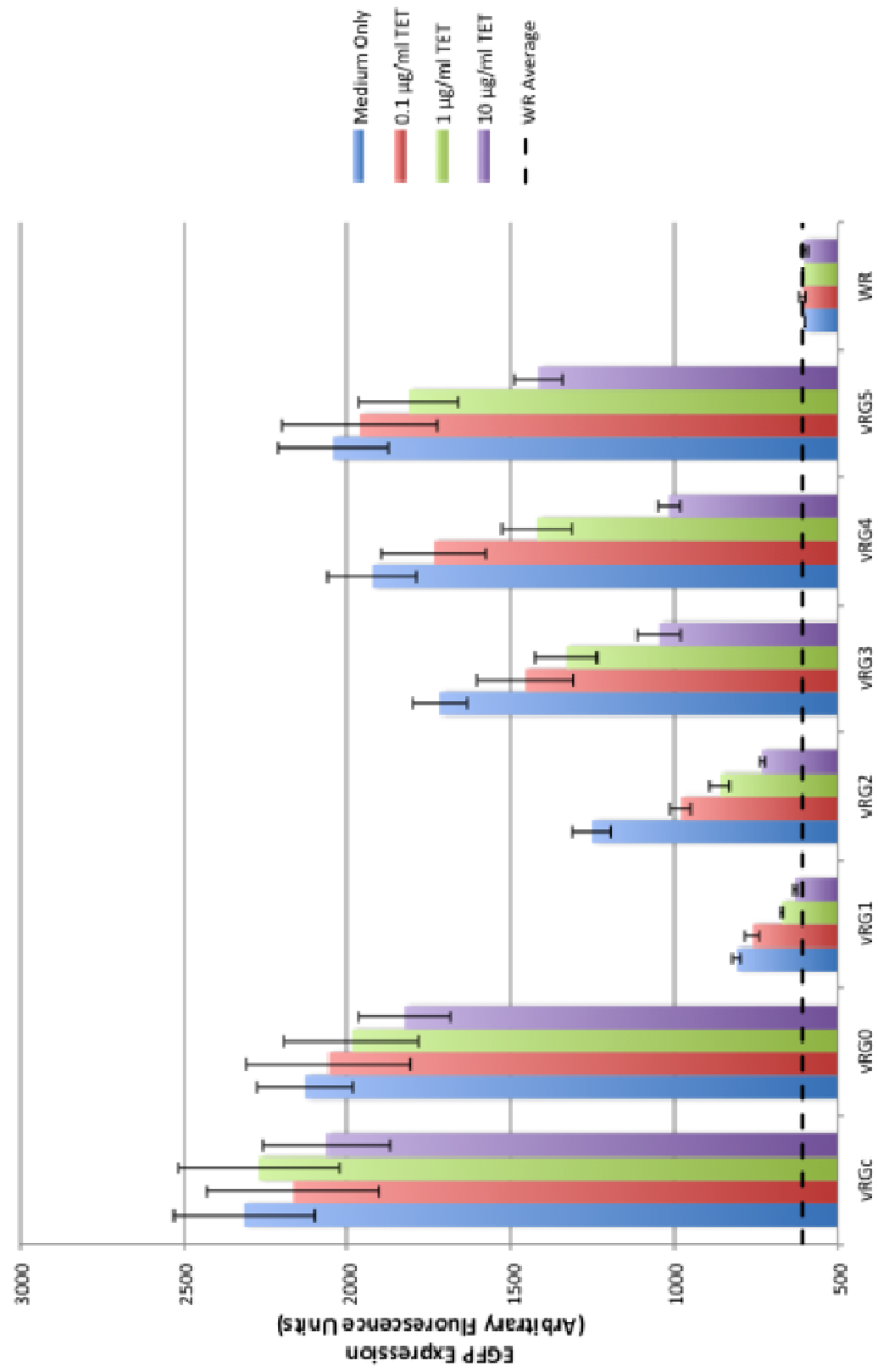


FIGURE 3.12. EGFP expression is repressed in the presence of increasing concentrations of DOX when measured by a multiwell fluorescence reader. In this preliminary study, BS-C-1 cells were infected at an MOI of 1 with the VACVs in the absence or presence of DOX and 2 DPI cells were fixed and quantified with a fluorometer. EGFP expression levels were repressed in the presence of increasing concentrations of DOX. Error bars are SEM, n=2.

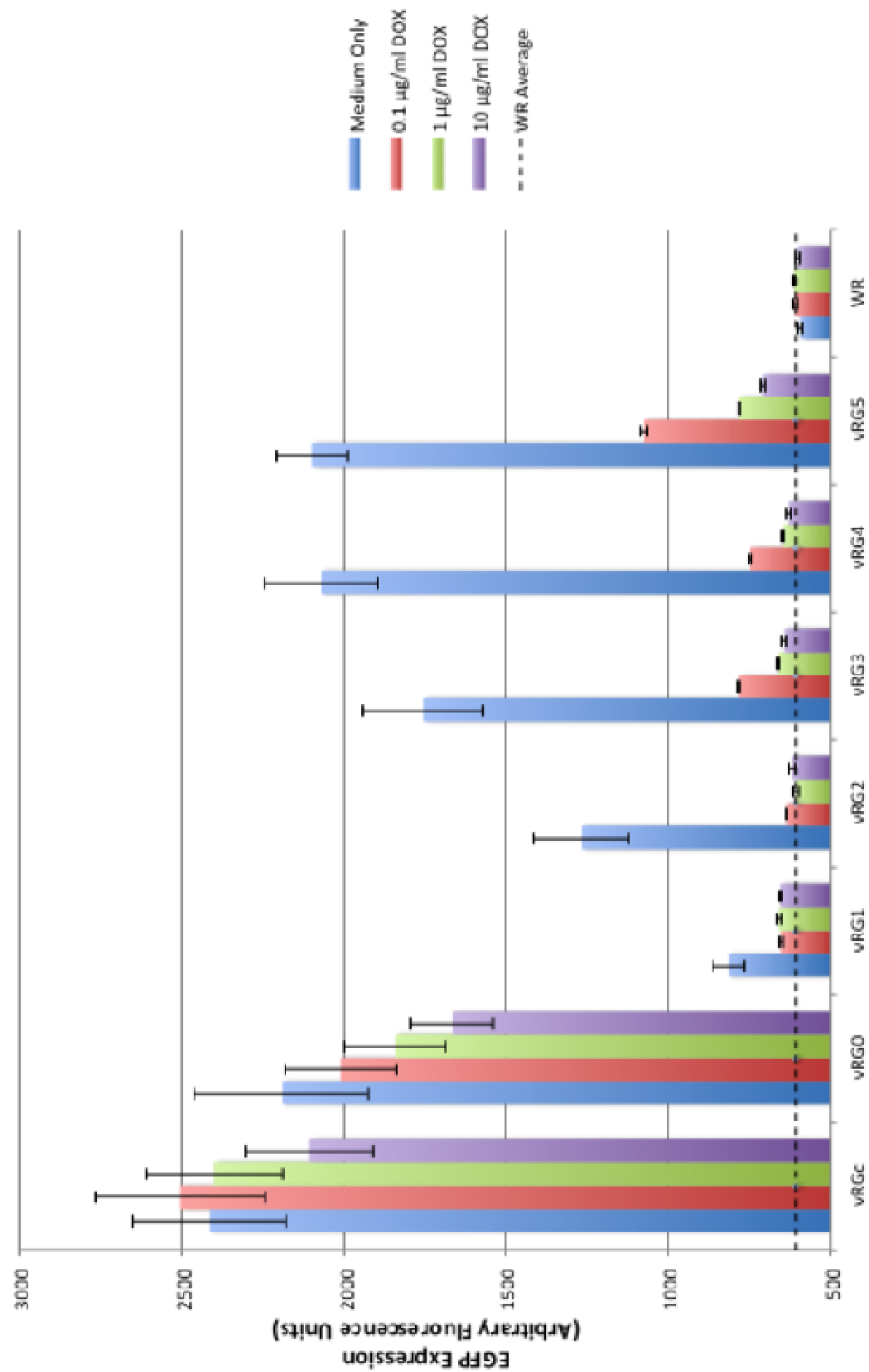


FIGURE 3.13. ATC represses EGFP expression in a dose response manner. BS-C-1 cells were infected at an MOI of 1 with the VACVs in the absence or presence of ATC and 2 DPI cells were fixed and quantified with a multiwell fluorescence reader. EGFP expression levels were repressed in the presence of increasing concentrations of ATC. Error bars are SEM, n=2.

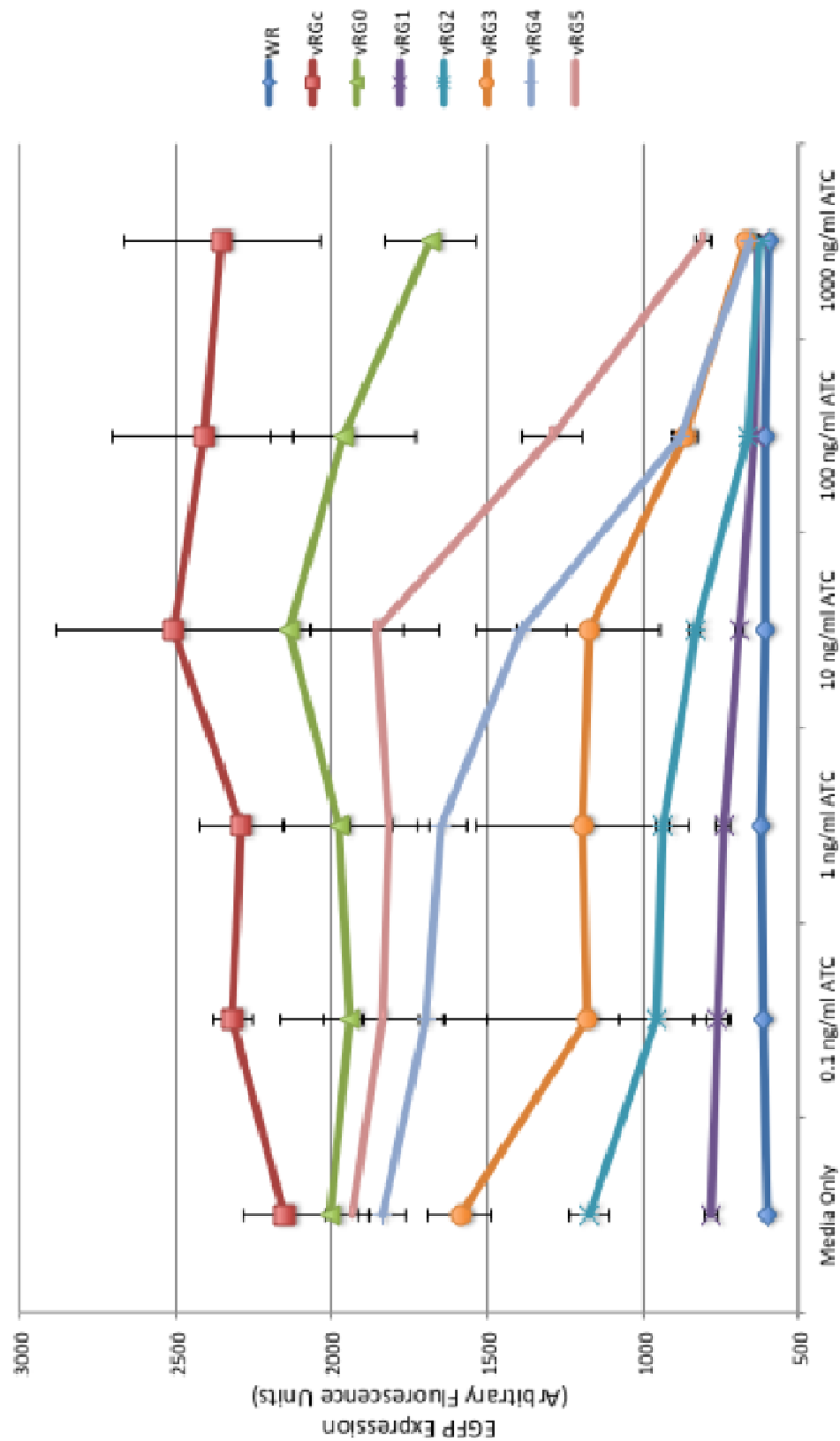


FIGURE 3.14. TET represses EGFP expression in a dose response manner. BS-C-1 cells were infected at an MOI of 1 with the VACVs in the absence or presence of TET and 2 DPI cells were fixed and quantified with a multiwell fluorescence reader. EGFP expression levels were repressed in the presence of increasing concentrations of TET. Error bars are SEM, n=2.

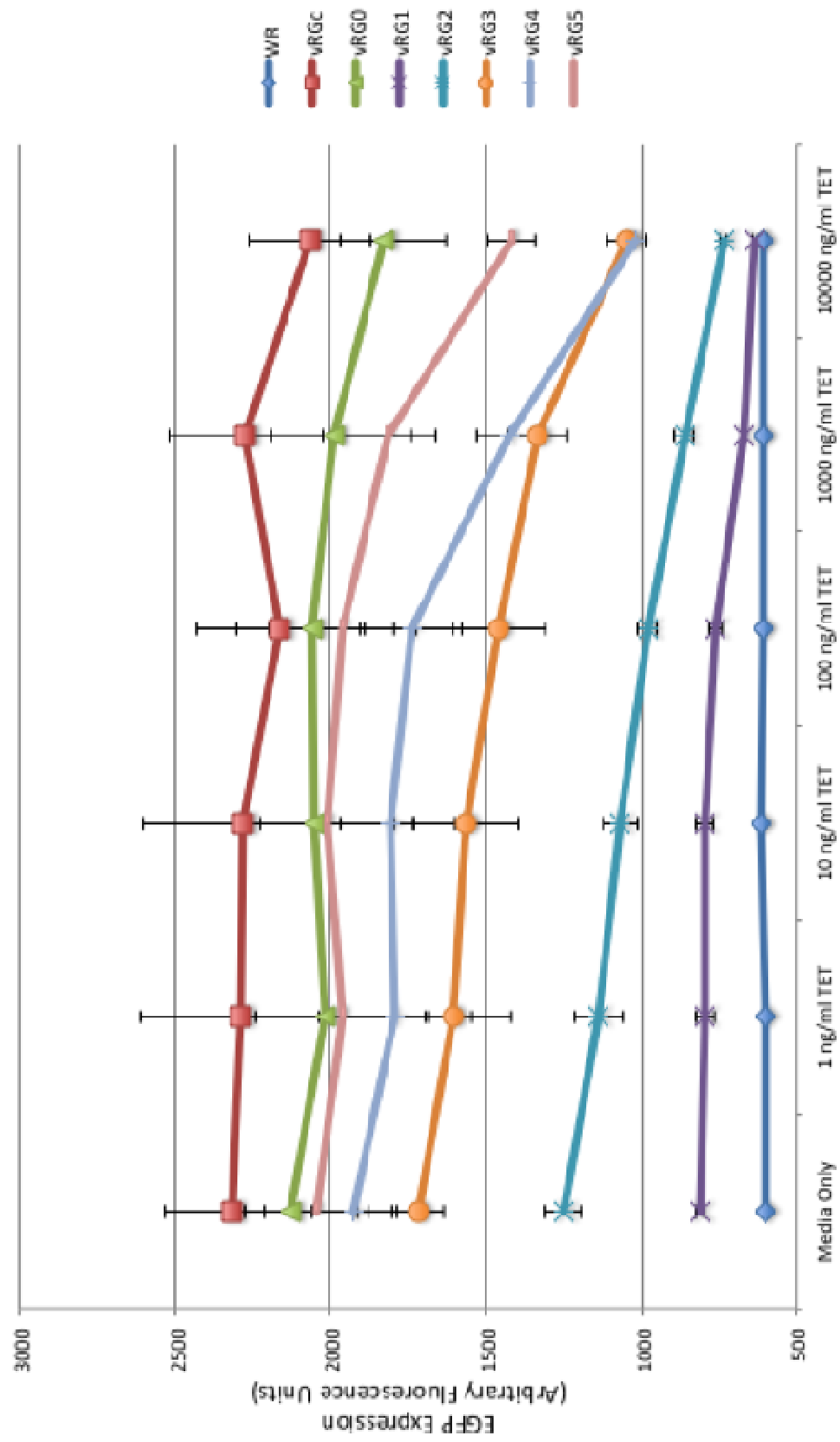


FIGURE 3.15. DOX represses EGFP expression in a dose response manner. BS-C-1 cells were infected at an MOI of 1 with the VACVs in the absence or presence of DOX and 2 DPI cells were fixed and quantified with a multiwell fluorescence reader. EGFP expression levels were repressed in the presence of increasing concentrations of DOX. Error bars are SEM, n=2.

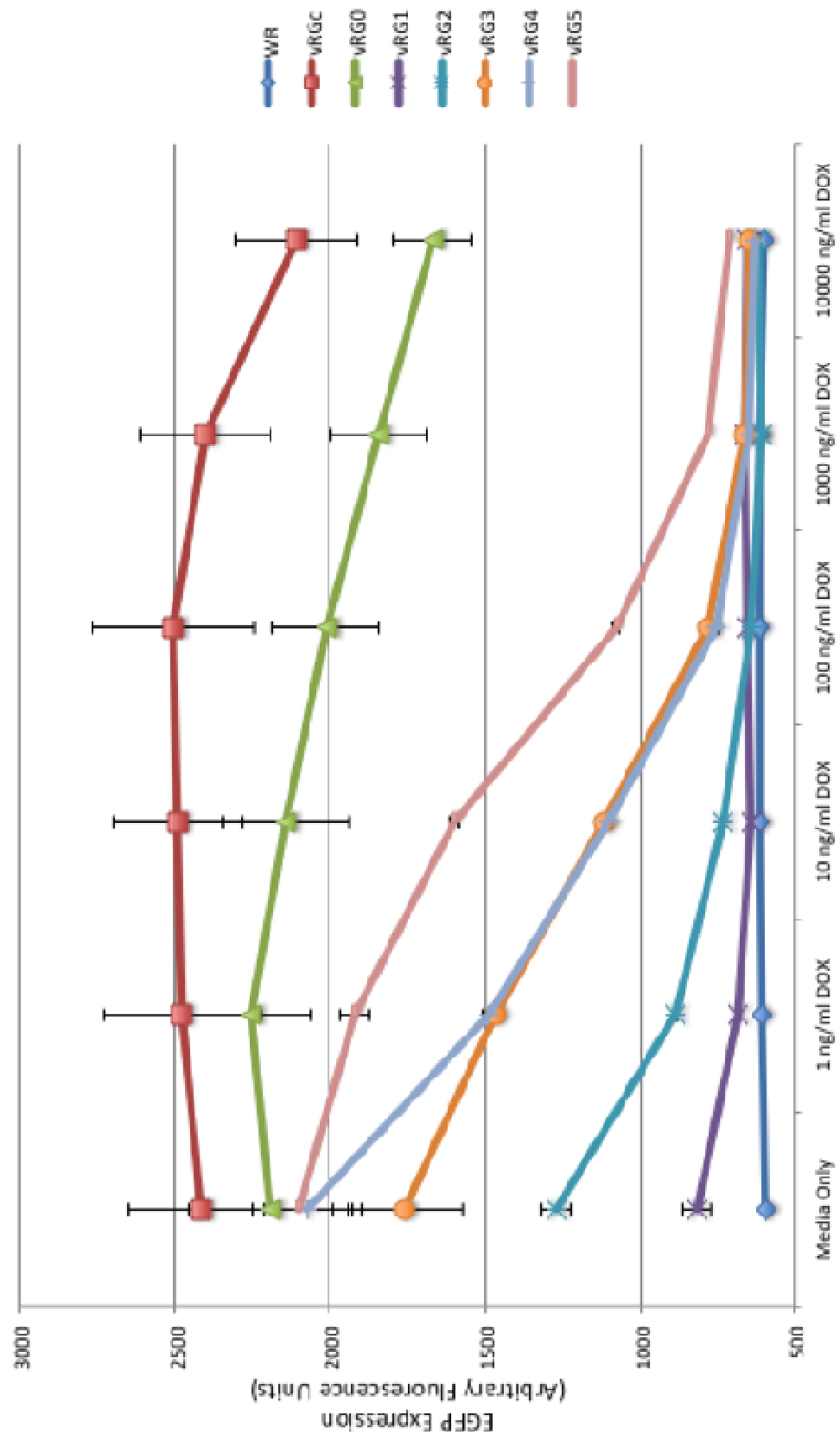


TABLE 3.1. RevTetR mutants used to generate the recombinant VACVs used in this study.

Recombinant VACV	revTetR	Mutations	References
vRG0	L17G	L17G	Scholz et al., 2004
vRG1	Original rtTA	E71K, D95N, L101S, G102D	Gossen et al., 1995
vRG2	Advanced rtTA (rtTA-M2)	S12G, E19G, A56P, D148E, H179R	Urlinger et al., 2000
vRG3	rtTA-S2	E19G, A56P, D148E, H179R	Urlinger et al., 2000
vRG4	rtTA-M1	S12G, E19G, A56P	Urlinger et al., 2000
vRG5	rtTA-19/56R	E19G, A56P	Urlinger et al., 2000

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3.7. REFERENCES

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